

Regulation of hippocampal synaptic transmission and receptor trafficking by adenosine in hypoxia and ischemia: role of protein phosphatases 1, 2A and 2B, casein kinase 2 (CK2), and equilibrative nucleoside transporters (ENTs).

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Abstract

The role of adenosine as an endogenous neuromodulator is well established, but the mechanism(s) mediating the extensive modulatory and regulatory actions of adenosine have not yet been fully elucidated. In fact, although adenosine, through activation of adenosine A1 and A2A receptors, has been demonstrated as neuroprotective or neurodegenerative, respectively, little is known about the mechanism by which adenosine mediates these actions. In the hippocampus, essential physiological processes rely on adenosine signaling, including regulation of long-term potentiation (LTP) and long-term depression (LTD). Neuromodulation by adenosine is dominantly inhibitory in the hippocampus, mediated by the abundant and high-affinity adenosine A1 receptor. In ischemia and hypoxia, A1 receptor activation induces rapid synaptic depression which is mediated by multiple signaling pathways including the induction of excitatory AMPA glutamate receptor internalization, which inhibits synaptic transmission in the hippocampus. Considerable effort has been devoted to investigating the role of adenosine in ischemic stroke, due to the fact that in cerebral ischemia or hypoxia, extracellular levels of adenosine increase dramatically. This thesis explores the functional consequences of adenosine signaling in hypoxia and ischemia, which mediate GluA1 AMPA receptor subunit internalization. Three major serine/threonine protein phosphatases (PPs), PP1, PP2A, and PP2B are investigated and shown to mediate A1 receptor-mediated GluA1 internalization in hypoxic conditions in the rat hippocampus. Further experiments demonstrate the role of adenosine A2A receptors in potentiating hippocampal synaptic transmission in reperfusion by increasing GluA1 surface expression through increased phosphorylation of regulatory C-terminal phosphorylation sites of GluA1. The mechanism of extracellular adenosine regulation by equilibrative nucleoside transporters (ENTs) and casein kinase 2 (CK2) are examined and shown to interact in hypoxia/reperfusion experiments on hippocampal slices. Finally, using a pial vessel disruption (PVD) permanent focal cortical ischemia stroke model, experiments demonstrate increased adenosine tone in the hippocampus, which mediates increased adenosine-induced synaptic depression. CK2 inhibition was also neuroprotective after 20min hypoxia. This shows that adenosine tone is increased in the hippocampus after a small cortical stroke, implying a potential global effect of focal ischemia. Together, these studies further reveal the paramount role of adenosine as a neuromodulator in the hippocampus during neuronal insults, furthering our understanding of the mechanism of neuronal death in hypoxic and ischemic conditions.

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Chapter 1: Introduction

1.1 Introduction

Stroke is becoming an increasingly common disease and has become a major healthcare issue. It is the leading cause of disability in North America and also the third leading cause of death (Marler et al., 2000). Stroke rehabilitation is a substantial, long-term cost on the healthcare system, and an improvement from current therapies is urgently needed (Pantoni, 2010). Numerous resources have been devoted to developing effective neuroprotective stroke therapies, with little success thus far in clinical trials (Gladstone et al., 2002; Ikonomidou and Turski, 2002; Ali et al., 2013). In fact, currently only one treatment has been approved for stroke, which is the use of *recombinant tissue plasminogen activator* (rt-PA) to resolve a stroke-causing vascular clot in the brain (Friedman et al., 1996). Unfortunately, rt-PA is a relatively ineffective therapy, working in only a small percentage of stroke patients (~3%), largely due to the limited time window for treatment (~90-180 minutes), and can induce intracranial hemorrhage in a small number of patients (Marler et al., 2000). There has also been question as to whether rt-PA was initially approved based on a misinterpretation of clinical trial data (Mann, 2002). Hence, characterization of new therapeutic targets for neuroprotective stroke therapy is critical.

To successfully define a therapeutic target for neuroprotection in neuronal insults such as stroke, an in-depth understanding of the cellular mechanisms contributing to neuronal damage vs. survival in stroke is necessary. A stroke can be classified as either *hemorrhagic*, where a blood vessel ruptures and bleeds into the skull, or *ischemic*, where there is a disruption to blood flow to tissue in the brain and account for about 85% of all stroke cases (Friedman et al., 1996; Lammie, 2000). Ischemic stroke is characterized by a reduction in blood flow to an area of the brain caused by a clot or stenosis (Marler et al., 2000). Reduced blood flow lowers oxygen and nutrient delivery to the ischemic brain tissue, inducing a number of compensatory mechanisms in an attempt to protect the neurons, such as the

activation of adenosine receptors, which is thought to be dominantly neuroprotective (Rudolphi et al., 1992; Wardas, 2002).

Two major components form in the brain due to ischemia: one being the ischemic core where brain tissue loses all perfusion and there is widespread cell death (necrosis). The other component is the ischemic penumbra, an area surrounding the ischemic core with insufficient perfusion but where the tissue may be salvageable if effective therapeutic interventions are developed. Neurons in the ischemic penumbra are susceptible to death through various mechanisms such as apoptosis (Sirén et al., 2001; Hardingham et al., 2002), excitotoxicity (Sommer et al., 2006), glial cell dysfunction (Largo et al., 1996), and oxidative stress (Murakami et al., 1998). The ischemic penumbra is currently a major target in stroke therapy development, with research focusing on rescuing penumbral tissue before the onset of neuronal death (Schiff and Somjen, 1987).

A major goal in this laboratory is to gain an improved understanding of the mechanism by which apoptosis occurs in ischemic tissue using hypoxia to simulate stroke conditions. Specifically, we examine the role of adenosine in the neuronal response to hypoxic conditions in the hippocampus. We recently implicated adenosine, an endogenous purine nucleoside, in the reduction of synaptic transmission in the hippocampus by regulation of the surface expression of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Chen et al., 2014). This thesis examines the adenosine-mediated internalization of the AMPA receptor subunit GluA1, which accompanies adenosine-induced synaptic depression in hypoxia. Additionally, we examine the role of casein kinase 2 (CK2) and equilibrative nucleoside transporters (ENTs) in the regulation of extracellular adenosine levels and their interaction with adenosine A1 receptors. Both protein phosphatase and CK2 drug modulators are then tested for potential neuroprotection to hippocampal slices subjected to a hypoxic insult (20min). Finally, using an *in vivo* pial vessel disruption focal cortical ischemia rat

model (PVD), we reveal evidence showing that focal cortical ischemia may have global effects in regions as far away from the lesion as the hippocampus. This chapter reviews important background literature about the key experiments performed in subsequent chapters.

1.2 Adenosine in the brain

Adenosine is a purine nucleoside required for cell survival. It is comprised of an adenine molecule attached to a ribose sugar, and has the potential to be phosphorylated. It plays an essential role in multiple cellular processes, such as cellular energy transfer and intracellular signaling (Collis and Hourani, 1993). In energy transfer processes, adenosine is conjugated with up to three phosphates, and is then known as adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP). Hydrolysis of these phosphorylated molecules releases energy that is utilized by almost all energy-requiring cellular processes in mammals, including widely in the brain (Zur Nedden et al., 2011). Due to the ubiquitous nature of adenosine, mammalian cells have developed the ability to use adenosine and other adenine nucleotides as signaling molecules (Collis and Hourani, 1993).

As a neuromodulator, adenosine modulates a wide array of physiological processes (Fredholm et al., 2000; Fredholm et al., 2005). A major consequence of adenosine signaling is the ability for adenosine to induce intracellular changes in metabolically stressed cells to restore energy balance, and has therefore been termed a retaliatory metabolite (Fredholm et al., 2005). Table 1.1 outlines examples of physiological consequences of adenosine signaling in the human body.

Table 1.1. Examples of the actions of adenosine throughout the body.

Physiological System	Examples of actions induced by adenosine
Central Nervous system	Modulation of neurotransmitter release ^{a,g} Sedation ^g Modulation of synaptic transmission ^{a,g} Modulation of locomotor activity ^{b,g}
Gastrointestinal Tract	Reduces stomach acid secretion Modulation of GI tone and secretions
Cardiovascular System	Vasoconstriction and vasodilation ^c Bradycardia Platelet inhibition Negative cardiac inotropy Angiogenesis ^h
Renal Function	Modulation of glomerular filtration ^b Mesangial cell contraction Antidiuresis Inhibition of renin release ^b
Metabolism	Reduces lipolysis Increases glucose uptake Increases insulin sensitivity Increases gluconeogenesis
Respiratory System	Bronchoconstriction - implicated in asthma Mucus secretion Respiratory depression
Immune System	Immunosuppression Neutrophil modulation Mast cell degranulation

Physiological functions reviewed in: a - (Cunha, 2005), b- (Collis and Hourani, 1993), c- (Feoktistov and Biaggioni, 1997), d- (Espinal et al., 1983), e- (Latini et al., 1996a), f- (Hershfield, 2005), g-(Fredholm et al., 2005), h-(Feoktistov et al., 2002)

Adenosine has been recognized as a major neuromodulator in both physiological and pathophysiological conditions (Rudolphi and Schubert, 1995; Fredholm et al., 2005). Recent advances in adenosine research have profoundly improved our understanding of the complex role that adenosine plays in the brain, yet a considerable amount remains unknown. Adenosine acts dominantly as an inhibitory neuromodulator, inducing reduced synaptic transmission when extracellular adenosine levels increase, which occurs during hypoxic or

ischemic events (Rudolphi and Schubert, 1995). A basal adenosine tone is maintained in physiological conditions and contributes to basal inhibitory tone in the hippocampus (Brundege and Dunwiddie, 1996). Substantial evidence suggests a neuroprotective role for adenosine in hypoxia/ischemia due to its ability to reduce synaptic transmission and prevent excitotoxic cell death (Rudolphi and Schubert, 1995; Sebastião et al., 2001; Cunha, 2005). Adenosine receptors comprise of a group of four receptor subtypes (A1, A2A, A2B, A3), which are differentially expressed in the brain, each having unique physiological functions (Dunwiddie and Masino, 2001).

1.3 Adenosine receptor subtypes and functions

Adenosine interacts with four G-protein-coupled adenosine receptor subtypes known as A1, A2A, A2B, and A3 (Collis and Hourani, 1993; Fredholm et al., 2000). These receptors are present in the plasma membrane of the cell and transduce intracellular signals through various G protein-coupled mechanisms. Each receptor subtype is encoded by different genes and have a conserved structure, forming a functional 7-transmembrane region receptor (Collis and Hourani, 1993). Adenosine receptors are differentially expressed in different cell types in the body, with high levels of expression in the brain. Coupling to different G-proteins and different affinities for adenosine also differentiate the actions of each receptor subtype. Of particular interest in the CNS are the A1 and A2A receptors, which have a multitude of regulatory roles that appear to be largely brain region-specific (Fredholm et al., 2005). For example, in areas associated with learning and memory, adenosine has been implicated in the modulation of synaptic plasticity (de Mendonca and Ribeiro, 1996). Table 1.2 outlines the four adenosine receptor subtypes, their affinity for adenosine, and their distribution in the brain.

Table 1.2. Adenosine receptor subtypes with their affinity for adenosine and expression distribution in the brain. Adapted from (Dunwiddie and Masino, 2001).

Receptor Subtype	Affinity for Adenosine	Distribution in the brain
A1	~70nM	Most abundantly expressed throughout the brain
A2A	~150nM	Most abundant in striatum, olfactory tubercle, and nucleus accumbens
A2B	~5100nM	Expressed in small amounts in all brain areas
A3	~6500nM	Expressed in the brain in significant amounts

1.3.1 The adenosine A1 receptor

The adenosine A1 receptor (A1R) is abundantly expressed in the brain (Latini et al., 1996a; Ferré et al., 1997). A1Rs have been implicated in the inhibition of neuronal activity, shown to be coupled to reduced neurotransmitter release (Fredholm and Dunwiddie, 1988), activation of hyperpolarizing K⁺ currents (Trussell and Jackson, 1985), inhibition of Ca²⁺ currents (MacDonald et al., 1986), induction of intracellular protein kinase and phosphatase activity (Brust et al., 2006; Brust et al., 2007), and the reduction of excitatory AMPA receptor surface expression (Chen et al., 2014). Adenosine A1 receptor activity has been linked to inhibition of multiple neurotransmitter systems including glutamate, γ -aminobutyric acid (GABA), acetylcholine (Ach), norepinephrine (NE), serotonin (5-HT), and dopamine (DA) release (Fredholm and Dunwiddie, 1988; Dunwiddie and Masino, 2001). One of the most important neuromodulatory actions of the A1R in the hippocampus is its inhibitory action on excitatory glutamatergic neurotransmission where synaptic activity can be almost entirely blocked when extracellular adenosine concentrations rise (Dunwiddie, 1980; Kocsis et al., 1984).

1.3.2 The adenosine A2A receptor

The excitatory adenosine A2A receptor is expressed to a lower degree compared to the A1R in most parts of the brain. However, in the striatum, olfactory tubercle, and nucleus

accumbens, A2ARs are highly expressed (Collis and Hourani, 1993). A2ARs generally have antagonizing actions to A1Rs, and are implicated in multiple mechanisms to increase neuronal activity (Costenla et al., 2011; Dias et al., 2012), for example, by activating Adenylyl cyclase, increasing cAMP and activating PKA, which has been shown to increase GluA1 AMPA receptor subunit activation (Dias et al., 2012). Activation of A2ARs has also been shown to increase presynaptic glutamate release (Rebola et al., 2008). An important attribute of the A2AR is that they have a lower affinity for adenosine than A1Rs, which is why the dominant actions of adenosine in the hippocampus are inhibitory (Alzheimer et al., 1991a). Activation of A2ARs has been associated with the modulation of processes such as mood (Shen and Chen, 2009), learning and memory (Costenla et al., 2011), and neurodegeneration in stroke or hypoxia, as well as other neurodegenerative diseases (Cunha, 2005; Pugliese et al., 2009).

1.3.3 The adenosine A2B receptor

The adenosine A2B receptor was first identified as a distinct adenosine receptor subtype following the observation that although it signals through the activation of adenylyl cyclase similar to the A2A receptor, it has a much lower affinity for adenosine and is widely expressed throughout the brain (Latini et al., 1996a; Dunwiddie and Masino, 2001). The A2B receptor, has been less well-characterized in the CNS compared to A1 and A2A receptors, with much of its physiological role unknown (Dunwiddie and Masino 2001). A notable action of A2B receptor activation in the hippocampus is positive modulation of Ca^{2+} levels and increases the activity of P-type Ca^{2+} currents in CA3 pyramidal neurons (Collis and Hourani, 1993; Feoktistov and Biaggioni, 1997). Although this is one of few roles known for A2B receptors, their low affinity for adenosine (See table 1.2) could indicate their activation occurs only when there is a drastic increase in adenosine time, such as in pathological cell stress. Unfortunately, there is a current lack of an effective A2B receptor-specific

pharmacological agents which hinders an increased detailed understanding of this receptor subtype (Feoktistov & Biaggioni 1997). More work is required to further elucidate the physiological role of A2BRs in the CNS.

1.3.4 The adenosine A3 receptor

Finally, the adenosine A3 receptor is expressed in significant amounts throughout the brain (Zhou et al., 1992; De et al., 1993), although the A3R has the lowest affinity for adenosine compared to the other three receptor subtypes and therefore does not appear to significantly modulate synaptic function (Collis and Hourani, 1993). The highest amount of A3R mRNA is in the hippocampus and cerebellum (De et al., 1993), indicating higher receptor expression in these two important areas. A3R activation is associated with phospholipase C activation and increased inositol phosphate levels in the brain (Abbracchio et al., 1995). A3R activation using selective agonists reduces locomotor activity, suggesting a possible inhibitory role in the brain (Jacobson et al., 1993). A3Rs have been reported to act to uncouple A1Rs and metabotropic glutamate receptors (mGluRs) through a protein kinase C pathway (Dunwiddie et al 1997a, Macek et al 1998). This suggests a role for A3 receptors in the modulation of different G protein-coupled receptors.

Table 1.3. Summary of notable modulatory actions of adenosine receptor subtypes in the brain (Collis and Hourani, 1993; Dunwiddie and Masino, 2001; Chen et al., 2014).

Receptor Subtype	Coupled G-protein	Actions in the brain
A1	G _i and G _o	Inhibits neurotransmitter release Reduces AMPA receptor surface expression Activates K ⁺ channels Inhibits Ca ²⁺ channels
A2A	G _s and G _{olf}	Increases neurotransmitter release Increases Ca ²⁺ -permeable AMPA receptor expression Modulates locomotor activity
A2B	G _s	Increases cAMP Potentiates P-type Ca ²⁺ channels
A3	G _i and G _q	Increases excitatory release of aspartate Depress locomotor activity Causes A1R desensitization in the hippocampus

Table 1.3 summarizes some of major known roles of the four adenosine receptor subtypes in the brain. Due to differential tissue and cell type expression of the four adenosine receptor subtypes and uniformity of each receptor subtype throughout the body, development of drugs that target adenosine receptors in specific tissues has been a major challenge for researchers (Dunwiddie and Masino, 2001). Although each receptor subtype differs in adenosine affinity, G-protein coupling, and signal transduction mechanisms (Shryock et al., 1998; Linden, 2001), it is difficult to target adenosine receptors themselves as therapeutic targets. Instead, targeting intracellular adenosine receptor-mediated effects may prove effective in defining therapeutic targets for neuroprotection.

1.4 Adenosine-mediated neuromodulation in the hippocampus

In the hippocampus, adenosine has a dominantly inhibitory role due to the high levels of high-affinity A1 receptor expression (Ochiishi et al., 1999). Adenosine has been shown to modulate learning and memory functions including long-term potentiation (LTP) and long-

term depression (LTD) (Alzheimer et al., 1991b; Zhang and Schmidt, 1998; de Mendonca and Ribeiro, 2001). A major mechanism by which adenosine modulates synaptic transmission in both physiological and pathophysiological conditions is through modulation of excitatory glutamatergic neurotransmission (Rebola et al., 2005). Glutamate is an extremely important excitatory neurotransmitter that induces synaptic transmission through its action on glutamate receptors, which are separated into two broad groups: metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors. Ionotropic glutamate receptors include the ligand-gated AMPA, NMDA and kainate receptors (Nakanishi, 1992). AMPA receptors (AMPA receptors) mediate fast excitatory synaptic transmission, whereas NMDA receptors are known to mediate slower excitatory synaptic transmission (Lu et al., 1996).

In ischemia, a major cause of neuronal death is through excess excitatory neurotransmission, known as excitotoxicity. Both NMDA and AMPA receptors are known to play a role in excitotoxicity in the hippocampus (Lu et al., 1996; Kristensen et al., 2001). NMDA receptors have been studied extensively as neuroprotective drug targets for ischemia, but unfortunately NMDA receptor antagonists were proven ineffective as neuroprotectants in clinical trials (Ikonomidou and Turski, 2002). Because of this, focus has shifted to AMPA receptors as potential drug targets.

1.4.1 AMPA receptors

AMPA receptors (AMPA receptors) are ligand-gated ion channels that mediate the majority of fast excitatory neurotransmission in the brain (Burnashev et al., 1992; Martin et al., 1993). Functional AMPARs form tetramers that form a ‘dimer-of-dimers’ of four subunits, GluA1-4 (Nakagawa et al., 2005), forming Na^+ -, and sometimes Ca^{2+} -selective ion channels (Burnashev et al., 1992). Each AMPAR subunit has three transmembrane domains (TM1,3,4) and one re-entrant loop (TM2) containing a glutamine/arginine (Q/R) editing site where glutamine is posttranslationally replaced with arginine in GluA2 subunits, which renders

them impermeable to Ca^{2+} (Martin et al., 1993; Gu et al., 1996). This is particularly important because AMPARs with GluA2 are Ca^{2+} -impermeable, whereas GluA2-lacking AMPARs are permeable to Ca^{2+} (Burnashev et al., 1992; Greger et al., 2003).. Binding of glutamate to the extracellular ligand-binding site induces transient channel opening, inducing AMPAR-mediated currents (Nakagawa et al., 2005). Tetrameric GluA2-containing AMPARs are most abundant in many cell types, with GluA2-lacking AMPARs, which are dominantly GluA1 homomers and mediate transient increases in Ca^{2+} permeability, an essential function for example in LTP induction (Plant et al., 2006; Liu and Zukin, 2007).

We recently showed that GluA1 and GluA2 subunit-containing AMPAR surface expression is significantly decreased in the hippocampus after adenosine A1 receptor activation with the agonist *N*⁶-cyclopentyladenosine (CPA) (Chen et al., 2014). Along with showing a physical and functional interaction between A1Rs with GluA1 and GluA2 AMPAR subunits, we show that A1Rs induce endocytosis of these AMPAR subunits, which is differentially regulated by p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), protein phosphatase 2A (PP2A), and other potential intracellular second messenger proteins (Chen et al., 2014).

1.4.2 Calcium-permeable AMPA receptors and GluA1 regulation

Calcium permeability is a crucial attribute of GluA2-lacking AMPARs. Dysfunction in the regulation of AMPAR surface expression has been implicated in neurological diseases and neurodegeneration (Vandenberghe et al., 2000; Noh et al., 2005), and therefore, the tight regulation of AMPAR surface expression is essential. For example, in ischemic conditions with increased extracellular adenosine, calcium-permeable AMPARs (CP-AMPA) have been shown to interact with adenosine A2ARs, whereby A2AR activation induces increased GluA1 surface expression through activating protein kinase A (PKA) (Dias et al., 2012). This A2AR-induced increased GluA1 surface expression has been associated with Ca^{2+} -induced

excitotoxicity in hippocampal neurons (Noh et al., 2005). To modulate the trafficking and function of GluA1, there are multiple phosphorylation, palmitoylation, and ubiquitination sites present on intracellular GluA1 residues (Lee et al., 2010). This thesis explores two GluA1 phosphorylation sites, Ser831 and Ser845, with focus on protein phosphatase actions at these two serine residues. Table 1.4 outlines all currently known GluA1 phosphorylation sites and functions that they have been shown to modulate.

Table 1.4. Currently known phosphorylation sites for GluA1. Data from (Roche et al., 1996; Delgado et al., 2007; Clem and Huganir, 2010; Lee et al., 2010; Ai et al., 2011; Lu and Roche, 2012).

Residue	Kinase?	Known Function
Ser831	PKC/CaMKII	modulates AMPAR currents induction of LTP
Ser845	PKA	controls single channel conductance modulates AMPAR excitation critical for LTD expression
Ser818	PKC	enhances mean open probability of GluA1 homomers promotes GluA1 synaptic incorporation
Ser567	CaMKII	negatively modulates Loop1-dependent AMPAR synaptic targeting
Thr840	PKC	reduced pT840 important in LTD

PKA, a well-known serine/threonine protein kinase, phosphorylates GluA1 at a C-terminal serine residue, Ser845, which, among other things (Table 1.4), induces increased GluA1 surface expression (Oh et al., 2006). Another serine residue, Ser831, a target of protein kinase C (PKC) and calcium/calmodulin protein kinase II (CaMKII), has also been shown to increase surface expression and single-channel conductance of the AMPAR channel (Barria et al., 1997). Internalization of GluA1 through an endocytic pathway has been shown to occur

by dephosphorylation of GluA1 at Ser845 (Ehlers, 2000). Thus, dynamic regulation of these two serine phosphorylation sites is a major mechanism of GluA1 regulation (Roche et al., 1996), and therefore of the relative Ca^{2+} permeability at the synapse.

1.5 Regulation of extracellular adenosine

Extracellular concentrations of adenosine are tightly regulated, and changes in adenosine concentration can induce widespread effects. One mechanism through which adenosine is transported both directions across cell plasma membranes is through ENTs, which are widely expressed in the hippocampus (Baldwin et al., 2004). In ischemia/hypoxia, the concentration of extracellular adenosine increase dramatically up to 100 times normal levels (Dale et al., 2000), with two possible sources: adenosine release from ischemic cells into the extracellular space, and extracellular ATP metabolism (Benarroch, 2008). As discussed above, this increased extracellular adenosine is dominantly inhibitory in the hippocampus (Yoon and Rothman, 1991).

Two classes of nucleoside transporters exist in mammalian cells: *concentrative nucleoside transporter* (CNT) nucleoside pumps and *equilibrative nucleoside transporters* (ENTs) (Baldwin et al., 2004; Kong et al., 2004). ENTs are of particular interest due reports implicating ENT modulation as a contributing factor in ischemia/hypoxia-induced neurodegeneration (Ribeiro, 2005). ENTs are a subfamily of nucleoside transporters and are classified into four subtypes (ENT1-4), with ENT1 and ENT2 most widely expressed throughout the body including high levels of expression in pyramidal neurons in the hippocampus (Anderson et al., 1999). An important potential modulator of ENT1 expression and function is CK2, which targets two potential consensus sequences on the intracellular C-terminal of ENT1 (Stolk et al., 2005; Bone et al., 2007). Phosphorylation of ENT1 increases its surface expression, which have been shown to be mediated by CK2 (Stolk et al., 2005).

This may have important implications during hypoxia/ischemia. Regulation of extracellular adenosine in ischemic or hypoxic conditions by ENTs is mediated by altering the surface expression of ENTs (Chu et al., 2013). It was shown by Zhang et al. (2011) that neuronal ENT1 reduced hypoxia/ischemia-induced increase extracellular adenosine and suggested ENT1 as a potential therapeutic target for neuroprotection (Zhang et al., 2011).

1.6 Research objectives and Hypotheses

The major objectives of this thesis are to investigate the role of protein phosphatases (PP1/PP2A/PP2B) and CK2 in A1R- and hypoxia-induced synaptic depression and/or neuronal damage. In neuronal insults, increased extracellular adenosine induces increased adenosine receptor activation (Dale et al., 2000). The dominantly expressed high-affinity A1 receptor activation has been shown to induce significant synaptic depression through both presynaptic and postsynaptic modulatory mechanisms (Brust et al., 2006; Brust et al., 2007; Chen et al., 2014). Of particular importance, we recently showed that prolonged adenosine A1 receptor activation causes a phenomenon we termed APSD during A1R agonist drug washout. APSD was observed when fEPSPs in CA1 of rat hippocampal slices treated with CPA for 30min failed to recover to baseline levels even after 1 hour of agonist washout (Chen et al., 2014). This thesis aims to increase current understanding of the mechanism by which adenosine induces alterations in both AMPAR and ENT expression and function, and the implications of altered adenosine tone in the hippocampus caused by hypoxia and in small cortical PVD lesion *in vivo*. The following chapters show the results of the research that was carried out over the course of my Master's degree research.

The studies performed in Chapter 2 investigate a potential role for three major serine/threonine protein phosphatases in adenosine-mediated regulation of AMPAR surface expression: protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and protein

phosphatase 2B (PP2B), also called calcineurin. All three phosphatases have been shown to mediate multiple functions in the brain such as LTP and LTD (Belmeguenai and Hansel, 2005), different neuroprotective pathways (Don Yi and Simpkins, 2008; Hédou et al., 2008), and regulation of excitatory neurotransmission (Ito and Karachot, 1992; Launey et al., 2004). Results of these studies indicate a novel role for PP1, PP2A and PP2B in the regulation of A1R-induced synaptic depression in hypoxia via their dephosphorylation of GluA1 AMPAR subunits, which induce GluA1 endocytosis. After a 20 minute hypoxic insult, significant synaptic potentiation was induced by A2AR activation, which induces PKA-mediated GluA1 phosphorylation, causing increased GluA1 surface expression (Dias et al., 2012). Using protein phosphatase inhibitor drug treatments, we showed that A1R-induced synaptic depression can be partially ameliorated by protein phosphatase inhibitor pre-treatments, and also shows that these drugs afford neuroprotection to hippocampal slices after prolonged hypoxia.

Chapter 3 explores a functional interaction between ENT1, casein kinase 2 (CK2) and A1Rs in the functional consequences of modulating the function of these proteins in hypoxia. Inhibition of ENT1 and ENT2 induces increased extracellular adenosine tone, which was also shown after CK2 inhibition. This indicates that ENTs, of which ENT1 has CK2 target consensus amino acid sequences (Kiss et al., 2000; Handa et al., 2001), interact with CK2, which regulates ENT function in CA1 region of the rat hippocampus. When hippocampal slices were subjected to a 20min hypoxic insult, ENT1/2 inhibition prevented both hypoxia-induced synaptic depression and normoxic reperfusion-induced synaptic potentiation. CK2 inhibition also reduced hypoxia-induced synaptic depression and prevented reperfusion-induced synaptic potentiation (APHP). Using 20min hypoxia, treatment of hippocampal slices with CK2 inhibitors proved to have neuroprotective abilities in hypoxia. Finally, 48 hours after rats were given a permanent cortical ischemia pial vessel disruption (PVD)

surgery, hippocampal slices were shown to have increased inhibitory tone compared to sham-operated animals. Overall, these studies implicate three major protein phosphatases, PP1, PP2A and PP2B, and CK2 in the regulation of adenosine-mediated reactions to hypoxic conditions in the hippocampus. Based on these data, we propose these essential proteins as neuroprotective targets in ischemic conditions in the brain.

Chapter 2: Adenosine-mediated regulation of GluA1-containing AMPA receptors involves alterations of GluA1 Ser831 and Ser845 phosphorylation: protein phosphatases 1, 2A and 2B mediate adenosine A1 receptor-induced GluA1 endocytosis in rat hippocampal slices in hypoxia/reperfusion injury

2.1 Summary

Synaptic remodeling is a major mechanism of autoregulation in the hippocampus during both physiological situations and neuronal insults such as hypoxia and ischemia. Adenosine contributes to the modulation of neuronal insult-induced changes in the hippocampus, acting through both the inhibitory A1 receptor and excitatory A2A receptor, with A1Rs having a higher affinity for adenosine than A2ARs (See Table 1.1). We recently showed a novel mechanism by which adenosine A1 receptor activation induces excitatory GluA1 and GluA2 AMPA receptor subunit endocytosis and *adenosine-induced persistent synaptic depression* (APSD) in CA1 of rat hippocampal slices. The present study investigates the mechanism of adenosine-mediated GluA1 surface expression regulation by protein phosphatase 1 (PP1), 2A (PP2A), and 2B (PP2B). Using biotinylation Western blotting of hippocampal lysates and fEPSP electrophysiology experiments in CA1 of rat hippocampal slices, we show that A1R activation induces rapid GluA1 endocytosis and APSD, which was reduced by PP1, PP2A and PP2B inhibition. Additionally, the phosphorylation of two GluA1 serine residues, Ser831 and Ser845, is reduced after prolonged A1 receptor activation. Inhibition of PP1/PP2A with okadaic acid (20nM), PP2A with fostriecin (20nM), PP1 with tautomycin (20nM) and PP2B with FK506 (50nM), differentially prevented the reduction of phosphorylation at Ser831 and Ser845. In hypoxia/reperfusion experiments, adenosine induces rapid synaptic depression in CA1 followed by significant *adenosine-induced post-hypoxic potentiation* (APHP), which we found was induced by adenosine A2A receptor activation causing increased PKA activity, which increases GluA1 surface expression. A2AR inhibition with the selective antagonist SCH442416 (5nM) prevented the induction of APHP. Finally, hippocampal slices treated with protein phosphatase inhibitors followed by 20min hypoxia showed significantly less propidium iodide staining in the hippocampus compared to slices subjected to hypoxia alone, which was an indication of neurodegeneration induced by

hypoxia. This suggests a neuroprotective role for the inhibition of protein phosphatases in hypoxic conditions, revealing these as potential therapeutic targets.

2.2 Introduction

Adenosine is an essential neuromodulator in the brain, and has been shown to play a role in a physiological processes including learning and memory (Hogan et al., 1998), and pathophysiological processes such as ischemic stroke (Rudolphi and Schubert, 1995) and Parkinson's disease (Chen et al., 2001). Although adenosine's role as an important neuromodulator has been established, the mechanism by which adenosine receptors modulate synaptic activity is less well understood. Of the four G-protein-coupled adenosine receptors (A1, A2A, A2B, and A3), the A1 and A2A receptors have the most pronounced and well-classified expression in the central nervous system (CNS) and have opposing inhibitory and excitatory actions, respectively (Sichardt and Nieber, 2007). In hypoxia or ischemia, extracellular adenosine increases up to 100-fold from extracellular ATP breakdown and adenosine extrusion from ischemic cells (Dale et al., 2000; Pearson et al., 2001). In fact, peripheral plasma adenosine has been shown to be elevated in humans up to 15 days after a stroke or transient ischemic attack (Laghi Pasini et al., 2000), consistent with increased adenosine in the brain. Induction of synaptic depression by A1R activation is thought to afford neuroprotection to ischemic cells by preventing excitotoxicity by reducing glutamate signaling caused by increased glutamate release that can occur in hypoxia/ischemia (Liu et al., 2006). The role of adenosine in the induction of hippocampal synaptic depression in ischemia/hypoxia through adenosine A1 receptor (A1R) activation is thought to be neuroprotective by preventing excitotoxicity both presynaptically by inhibiting glutamate release (Obrietan et al., 1995) and postsynaptically by reducing cellular excitability (Cunha, 2005).

We recently showed that A1R activation induces rapid clathrin-mediated AMPAR endocytosis of excitatory glutamatergic AMPA receptors (AMPA receptors) through both a physical and functional interaction (Chen et al., 2014). Notably, A1Rs interact with GluA1 and GluA2

AMPA subunits to induce AMPAR endocytosis mediated by p38 MAPK, JNK, and PP2A. We showed that p38 mitogen-activated protein kinase (MAPK) and c-Jun n-terminal kinase (JNK) inhibition prevented adenosine-induced GluA1- and GluA2-containing AMPAR internalization, although inhibition of p38 and JNK did not entirely prevent A1R-induced GluA1 internalization (Chen et al., 2014). We also showed that protein phosphatase 2A (PP2A) mediated adenosine-induced GluA2- and GluA1-containing AMPAR internalization where PP2A inhibition prevented A1R-induced AMPAR endocytosis. Additionally, prolonged A1R activation induced APSD in CA1 of rat hippocampus. In the current chapter, I further investigate the mechanism of A1R-induced AMPAR internalization, with specific focus on A1R-induced GluA1 subunit internalization.

GluA1 is known to play an essential role in fast excitatory synaptic transmission in the hippocampus, and are essential for the induction of certain forms of synaptic plasticity (Lee et al., 2010). GluA1 is also present in a majority of hippocampal neurons (Rogers et al., 1991). Of particular importance, AMPARs that lack the GluA2 subunit, the vast majority of those being GluA1 homotetramers, are permeable to Ca^{2+} , and their overexpression has been shown to play a role in ischemia-induced neuronal death (Kwak and Weiss, 2006). Accordingly, blockade of Ca^{2+} -permeable AMPARs (CP-AMPA) has been shown to reduce cerebral ischemia-induced neuronal death (Noh et al., 2005). It follows that A1R-induced GluA1 internalization is an endogenous protective mechanism to prevent acute excitotoxicity during neurotoxic stimuli such as in cerebral ischemia.

GluA1 surface expression is known to be regulated in part by the phosphorylation state of two major C-terminal serine residues of GluA1, Ser831 and Ser845, which control multiple functions of GluA1 such as surface expression, synaptic translocation, channel open probability, and channel conductance (Lee et al., 2003). Phosphorylation of Ser831 and Ser845 by PKC/CaMKII and PKA, respectively, have been shown to induce increased GluA1

surface expression and synaptic localization (Gu et al., 1996; Esteban et al., 2003; He et al., 2011). Dephosphorylation of Ser831 and Ser845 have also been shown to mediate a reduction in the surface expression of GluA1 (Beattie et al., 2000; Oh et al., 2006; Lee et al., 2010). We previously showed that A1R activation induced reduced phospho-Ser831 (pSer831) and pSer845 that accompanies A1R-induced GluA1 endocytosis (Chen et al., 2014). We hypothesized that the reduction in GluA1 surface expression was due to PP2A activation, which I show here mediated A1R-induced GluA1 endocytosis.

Due to the involvement of PP2A in A1R-induced GluA1 endocytosis (Chen et al., 2014), I examined an involvement of PP2A and other serine/threonine phosphatases in reducing the phosphorylation of Ser831 and Ser845. Three major serine/threonine phosphatases present in hippocampal neurons are protein phosphatase 1 (PP1), PP2A, and PP2B/calcineurin (Don Yi and Simpkins, 2008), all of which have increased activity in hypoxia and ischemia (Brust et al., 2006; Hédou et al., 2008; Shintani-Ishida and Yoshida, 2011). PP1, PP2A, and PP2B have been shown to play a role in different neuroprotective mechanisms and cellular responses to neuronal insult conditions, as well as AMPA and NMDA receptor modulation in the hippocampus (Beattie et al., 2000; Belmeguenai and Hansel, 2005; Don Yi and Simpkins, 2008; Hédou et al., 2008). A1R stimulation induces multiple intracellular signals, including the recruitment of PP2A to the cellular membrane of hippocampal neurons (Brust et al., 2006). We hypothesized that PP1, PP2A and PP2B activity mediates A1R-induced GluA1 dephosphorylation, which contributes to A1R-induced GluA1 internalization.

We tested this hypothesis using both fEPSP electrophysiology experiments and biotinylation Western blots of rat hippocampal slices with exposure to selective protein phosphatase inhibitors in the presence of N⁶-cyclopentyladenosine (CPA), a selective A1R agonist, and hypoxia/reperfusion conditions. We found that inhibition of PP1, PP2A, and PP2B reduced AP5D in CA1 of rat hippocampal slices, and that PP1, PP2A, and PP2B

inhibitor specifically prevented GluA1 endocytosis after CPA treatment. Following a 20min hypoxic insult, there was reduced A1R surface expression and increased A2AR surface expression. After a 45min normoxic washout/reperfusion period, slices showed significantly increased GluA1, but not GluA2, surface expression, indicating increased Ca^{2+} permeable AMPA receptors (CP-AMPA), which increase neuronal excitability (Liu and Zukin, 2007).. In CA1, hypoxia induced rapid synaptic depression whereas normoxic reperfusion induced significant synaptic potentiation, which was subsequently shown to involve A2AR activation. Treatment with protein phosphatase inhibitors also reduced hypoxia-induced hippocampal neuronal death, indicating that these may be therapeutic targets to reduce hypoxia-induced neuronal death. The results presented below indicate an important role for protein phosphatases in the acute neuronal response to hypoxic insult conditions in the vulnerable tissue of the hippocampus.

2.3 Methods

2.3.1 Ethics Statement

All animals were treated according to guidelines of the Canadian Council for Animal Care (CCAC) under the supervision of the University of Saskatchewan Committee on Animal Care and Supply under animal protocol approval number 20070090.

2.3.2 Hippocampal Slice Preparation

Young male Sprague Dawley rats (pn18-30 days) were anaesthetized with halothane and rapidly decapitated, with the brains immediately excised and submerged in oxygenated, ice-cold high-sucrose dissection medium containing the following: 87mM NaCl, 25mM NaHCO_3 , 25mM glucose, 75mM sucrose, 2.5mM KCl, 1.25mM NaH_2PO_4 , 7.0mM MgCl_2 , and 500 μM CaCl_2 (Brust et al., 2007). Hippocampal slices were taken at 400 μm thickness using a vibrating tissue slicer (VTS1200S, Vibram Instruments, Germany), and slicing was

performed in the same ice-cold oxygenated dissection medium as above. Slices were maintained for at least 1h at room temperature in oxygenated artificial cerebrospinal fluid (aCSF) containing the following: 126mM NaCl, 2.5mM KCl, 2.0mM MgCl₂, 1.25mM NaH₂PO₄, 26mM NaHCO₃, 10mM glucose, 2.0mM CaCl₂ (Brust et al., 2007). Oxygenation was accomplished by continually bubbling the solution with 95% O₂/5% CO₂.

2.3.3 Drug Treatments

Hippocampal slices were incubated in one of the following treatments: dimethyl sulfoxide (DMSO, vehicle control, Sigma, St. Louis, MO), fostriecin (20nM, Tocris, Bristol, UK), okadaic acid (3nM, 20nM, Tocris), tautomycetin (20nM, Tocris), and FK506 (50nM, Sigma). Fostriecin is a selective PP2A inhibitor, okadaic acid at the concentration used inhibits both PP1 and PP2A, tautomycetin is a selective PP1 inhibitor, and FK506 is a selective PP2B (also called calcineurin) inhibitor. N⁶-cyclopentyladenosine (CPA) was used as a selective adenosine A1R agonist at 500nM, and SCH442416 (Sigma) was used as a selective adenosine A2A receptor antagonist at 5nM. All drugs were dissolved in DMSO (Sigma) before being added to aCSF. The final concentration of DMSO was < 0.1% in each treatment.

2.3.4 Electrophysiology

Hippocampal slices were submerged in an electrophysiology recording chamber with constant perfusion of oxygenated aCSF (3ml/min) containing the same concentration of drug treatment as was used in drug pre-incubation. Field excitatory postsynaptic potentials (fEPSPs) were evoked by orthodromic stimulation of the Schaffer collateral pathway using a bipolar tungsten stimulating electrode (Axon Instruments, Foster City, CA). A pulled glass recording microelectrode filled with aCSF (resistance 1-3MΩ) was placed in CA1 stratum radiatum, which recorded fEPSPs induced by Schaffer collateral stimulation. Two different experimental protocols were used throughout the course of this research. In both protocols, fEPSPs were evoked for 0.1ms every 30s throughout each experiment. Slices were constantly

perfused with aCSF containing the appropriate protein phosphatase inhibitor drug treatments and concentration listed above. Baseline recordings were set to approximately 60% of maximal fEPSP values.

The first protocol was performed by recording a stable baseline recording of at least 20min followed by 30min perfusion of CPA (500nM) and a 60min washout period. The second protocol was performed with a 20min stable baseline recording followed by 20min hypoxia and 45min normoxic reperfusion. Hypoxia was achieved by bubbling aCSF with 95% N₂/5%CO₂ for at least 20min prior to the experiment and continually over the course of the experiment. The fEPSP signals were amplified 1000 times with an AC amplifier, band-pass filtered at 0.1-100Hz, digitized at 10kHz using a Digidata 1440A digitizer (Axon Instruments), and saved to a computer as a Clampex 9.0 (Axon Instruments) file. The collected fEPSP data were analyzed using Clampfit 9.0 (Axon Instruments). fEPSP slopes were normalized to the mean of the 10 sweeps (5min) immediately preceding drug perfusion. The mean normalized fEPSP slope was plotted as a function of time with error bars representing the standard error of the mean (SEM). Sample traces are the average of 5 sweeps from a representative recording from each treatment group. All histograms show the mean normalized percent inhibition from baseline \pm SEM. Statistical significance was assessed using one-way analysis of variance (ANOVA) with Tukey-Kramer *post-hoc* test.

2.3.5 Biochemistry

For biochemistry studies, one rat was used for each different treatment group in every experiment, with each brain's hippocampal slices distributed equally between treatment groups. A minimum of three of these experiments was performed prior to analysis. Following appropriate drug incubation in protein phosphatase inhibitors or DMSO (vehicle control), brain slices were treated with CPA (500nM) for 30min or hypoxia for 20min. CPA-treated slices were rapidly cooled to 4°C, and hypoxia-treated slices were either immediately cooled

or incubated in normoxic aCSF washout for 45min and then cooled to 4°C. To isolate cell surface proteins, slices were treated with NHS-SS-Biotin (1mg/ml, Thermo Scientific) for 1h at 4°C. The biotin reaction was quenched with glycine buffer containing 192mM Glycine and 25mM Tris (pH 8.3). Slices were then transferred to homogenization tubes and homogenized in lysis buffer (pH 8.0) containing 50mM Tris, 150mM NaCl, 1mM EDTA, 1mM NaF; and the following protease inhibitors: 1mM PMSF, 10g/L aprotinin, 10g/mL pepstatin A, 10g/mL leupeptin, 2mM Na₃VO₄, 20mM sodium pyrophosphate, 3mM benzamidine hydrochloride, and 4mM glycerol 2-phosphate with 1% NP-40 detergent.

A Bradford Assay was performed with DC Protein assay dye (Bio-Rad) to determine protein concentration in the lysates, and 500µg of protein lysate diluted in lysis buffer was loaded into Streptavidin agarose beads (Thermo Scientific) and rotated overnight at 4°C. The beads were then washed 4-6 times the next day with lysis buffer containing 0.1% NP-40 (otherwise same as above). The proteins were eluted by adding 50µl of 2X Laemmli sample buffer (Bio-Rad) and boiling the samples at 95 °C for 5min. Whole cell lysate samples of 50µg were eluted in 20µl of the same Laemmli buffer and boiled for 5min. Samples were loaded into 10% SDS-PAGE gels and run for 1h. Proteins were transferred from gels to Immobilon-P^{SO} transfer membranes (Millipore, 3h at 4°C). Membranes were then treated with primary antibodies overnight at 4°C as follows: GluA1 (Ms mAb, Millipore), GluA1-pSer831 (Millipore), GluA1-pSer845 (Millipore), GluA2 (Millipore), A1R (Millipore), and A2AR (Millipore). Membranes were then probed with appropriate secondary antibody and then ECL was performed (Santa-Cruz). Analysis was performed using Quantity One Basic (BioRad) and ImageJ (NIH, public domain). Graphs were constructed using GraphPad Prism 6.0 (GraphPad). Data are presented as mean ± SEM. Statistical significance was assessed using a one-way ANOVA test and a Tukey-Kramer multiple comparison test using GraphPad InStat version 3.0 (GraphPad).

2.3.6 Propidium Iodide Staining

Propidium iodide (PI) is an effective fluorescent marker for cell death due to the fact that it only enters and labels cells with disrupted plasma membranes. It produces strong red fluorescence when excited by green light. Propidium iodide staining and subsequent confocal imaging of rat hippocampal slices were used to examine the effect of protein phosphatase inhibitor treatments on cell survival after a 20min hypoxic insult. The methods used were adapted from Pugliese et al. (2009).

Following equilibration of hippocampal slices for 1h after slicing, the following drug treatments were added: okadaic acid (20nM), fostriecin (20nM), tautomycin (20nM), and FK506 (50nM). Slices were incubated in these treatments for 1h and were then subjected to a 20min hypoxic insult by replacing oxygenated aCSF with hypoxic aCSF that was bubbled with 95%N₂/5%CO₂ prior to, and continuously throughout the hypoxic insult. After 20min hypoxia, aCSF was replaced with normoxic aCSF and the slices were incubated at room temperature for 3h. During the final 1h incubation period, 5µg/ml propidium iodide (Sigma) was added to the aCSF. Following the incubation period, slices were rinsed thoroughly in aCSF and then fixed in 4% paraformaldehyde at 4°C overnight. The following day, slices were washed 3 x 15min in 1X PBS and then mounted on glass microscope slides (VWR) and sealed using Prolong Gold Antifade Reagent (Invitrogen). After the addition of PI, all subsequent procedures were performed in the dark to prevent photobleaching.

Hippocampal slices were imaged using a Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss, Germany) using green light (543nm) to induce PI fluorescence. The whole hippocampus was imaged in pieces using a 10x objective lens, and images of CA1 pyramidal neurons were obtained using the Zeiss Plan-Apochromat 63x/1.6 oil objective lens (Carl Zeiss). CA1 images were acquired as Z-stack images of 200µm depth into the

hippocampal slice with each Z-stack image taken at 2 μ m. Two Z-stack images were taken along CA1 for each slice and were averaged using densitometry analysis.

Data was collected using Zeiss Zen 2009 version 5.5 software (Carl Zeiss) and was analyzed using ImageJ. Z-stack images closest to the outer top and bottom of the hippocampal slices were not analyzed, as the neuronal damage in those areas was enhanced by the slicing procedure. The inner-most 20 μ m (~100 μ m into the slice) segments were combined as maximum intensity projections and intensities were compared between treatment groups using densitometry analysis. Collected densitometry data was normalized to time control slices that were treated along with each experiment. Data was graphed as a percentage of the time control value and analyzed for significance against this control value (100%). Full hippocampal images were assembled as montages of the entire hippocampal slice using Adobe Photoshop CS6 (Adobe Systems, Mountain View, CA).

2.4 Results

2.4.1 Inhibition of PP1, PP2A and PP2B prevented CPA-induced GluA1 internalization, whereas only PP2A inhibition prevented GluA2 internalization.

We first sought to test the effect of A1R activation in hippocampal slices that were pre-incubated in PP1, PP2A, and PP2B inhibitors for 1h followed by a 30min treatment with the A1R agonist CPA (500nM). We previously showed that the PP2A inhibitors okadaic acid (20nM) and fostriecin (20nM) prevented A1R-induced GluA1 and GluA2 internalization (Chen et al., 2014). In this experiment, we used both okadaic acid and fostriecin at the same concentrations used previously, and also used the PP1-selective inhibitor tautomycin (20nM) and the PP2B-selective inhibitor FK506 (50nM). Following a 30min treatment with 500nM CPA, slices were biotinylated and examined for AMPAR surface expression. Resultant Western blots show that hippocampal slices that were treated with tautomycin,

okadaic acid, fostriecin, and FK506 prevented the CPA-induced reduction in GluA1 surface expression (Figure 2.1A,B). Compared to CPA, which induced approximately 40% reduction in surface GluA1, phosphatase inhibitor treatments did not significantly alter GluA1 surface expression compared to control (100%). Conversely, CPA-induced GluA2 internalization was prevented by okadaic acid and fostriecin, but not tautomycin or FK506 (Figure 2.1C,D). Since the concentration of okadaic acid used may be selective for both PP2A and PP1, the effect of okadaic acid was compared to the effect of either fostriecin or tautomycin, which shows that the effect of okadaic acid on GluA2 internalization is likely due to PP2A alone. Additionally, PP2A has a higher affinity for okadaic acid compared to PP1 (Bialojan and Takai, 1988). These data indicate that PP2A is involved in A1R-induced GluA1 and GluA2 internalization, whereas PP1 and PP2B are involved in GluA1 internalization but not GluA2 internalization after A1R activation.

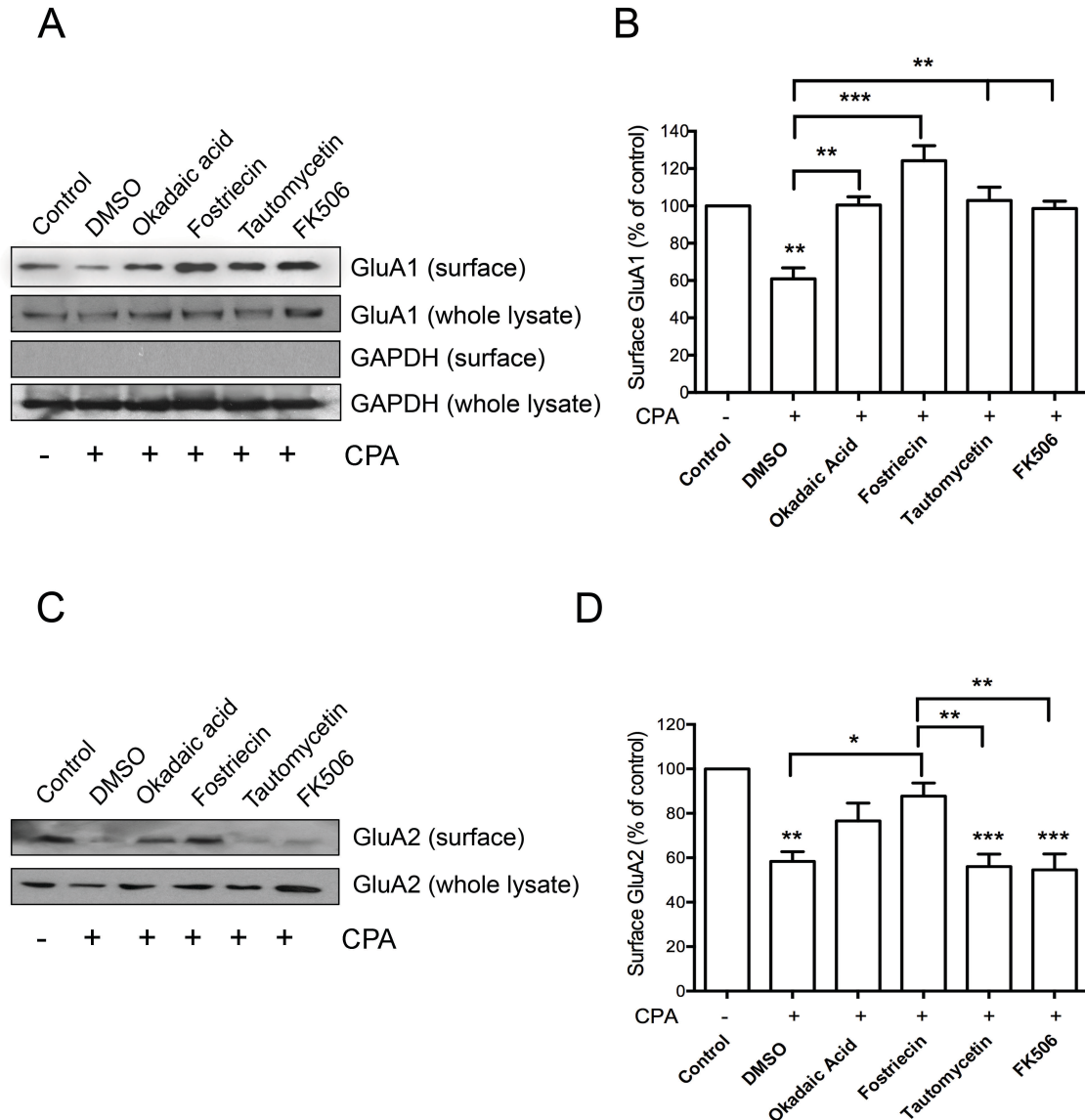


Figure 2.1. Protein phosphatase inhibitors reduced CPA-induced GluA1 internalization, and PP2A inhibition reduced CPA-induced GluA2 internalization. Biotinylated Western blots of hippocampal slices exposed to 30min CPA treatment (500nM) with pre-incubation in okadaic acid (20nM), fostriecin (20nM), tautomycetin (20nM), and FK506 (50nM). (A) Representative Western blots showing that GluA1 surface expression was reduced by approximately 40% after CPA treatment with no other drugs added, and treatment with protein phosphatase inhibitors for 1h prior to CPA treatment significantly reduced CPA-induced GluA1 internalization. Whole lysates probed for GluA1 showed constant levels

across all treatments, indicating that there is no significant intracellular degradation of GluA1 during CPA treatment. (B) Summary bar graph of results in A showing the percentage of control (100%) of the surface expression of GluA1. (C) Blots probed for GluA2 expression showed that CPA treatment reduced GluA2 surface expression in surface biotinylated blots. Treatment with okadaic acid and fostriecin, PP2A inhibitors, prevented CPA-induced GluA2 internalization, with fostriecin significantly preventing GluA2 internalization compared to DMSO. Treatment with tautomycin and FK506 did not prevent GluA2 internalization, indicating that PP1 and PP2B do not play a role in GluA2 surface regulation. Whole lysate levels of GluA2 did not significantly change between treatment groups, indicating there was no intracellular GluA2 degradation. (D) Summary bar chart for GluA2 expression from blots in C. Blots were normalized to intracellular GAPDH. Graphed values are shown as mean \pm SEM, n=4-6 independent experiments, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

2.4.2 Protein phosphatases 1, 2A, and 2B are involved in GluA1 phosphorylation at Ser831 and Ser845.

It has been shown that two C-terminus serine residues of GluA1, Ser831 and Ser845, are essential regulatory phosphorylation sites for GluA1 trafficking and function (Oh and Derkach, 2005). We recently showed that Ser831 and Ser845 phosphorylation was decreased after treatment with a relatively large dose of CPA (500nM) (Chen et al., 2014). To examine the role of PP1, PP2A and PP2B in A1R-mediated GluA1 dephosphorylation at Ser831 and Ser845, we tested the hypothesis that the phosphorylation state of Ser831 and Ser845 is modified by protein phosphatase inhibitor pre-incubation (1h, same concentrations as above) followed by 30min CPA treatment with protein phosphatase inhibitor pre-treatment (1h). Western blots probed for phosphorylated GluA1 at either Ser831 or Ser845 (pSer831 and pSer845) show that whole-cell levels of pSer831 and pSer845 were significantly reduced

after CPA treatment, by 25% and 35%, respectively (Figure 2.2A,B), which corresponds to the reduced GluA1 surface expression shown prior (Figure 2.1).

Hippocampal slices were treated with okadaic acid (20nM), fostriecin (20nM), tautomycetin (20nM) and FK506 (50nM) for one hour prior to administration of CPA (500nM) for 30 minutes. Figure 2.2 shows representative Western blots probed for whole lysate levels of pSer831 and pSer845, which were normalized to whole cell levels of GluA1. The level of pSer831 was significantly reduced following CPA treatment alone, and the reduction of pSer831 was prevented by tautomycetin (20nM) and FK506 (50nM) (Figure 2.2A), whereas okadaic acid and fostriecin did not significantly prevent the CPA-induced reduction of pSer831. CPA-induced reduction of Ser845, on the other hand, was prevented by okadaic acid and fostriecin whereas tautomycetin and FK506 did not significantly increase pSer845 compared to vehicle control (DMSO + CPA). These results indicate that Ser831, a PKC/CaMKII phosphorylation target (Roche et al., 1996), may be preferentially dephosphorylated by PP1 and PP2B whereas Ser845, a PKA target, could be preferentially targeted by PP2A. Taken together, these results reveal a differential regulation of Ser831 and Ser845 phosphorylation by these three phosphatases, which corresponds to CPA-induced GluA1 endocytosis (Figure 2.1).

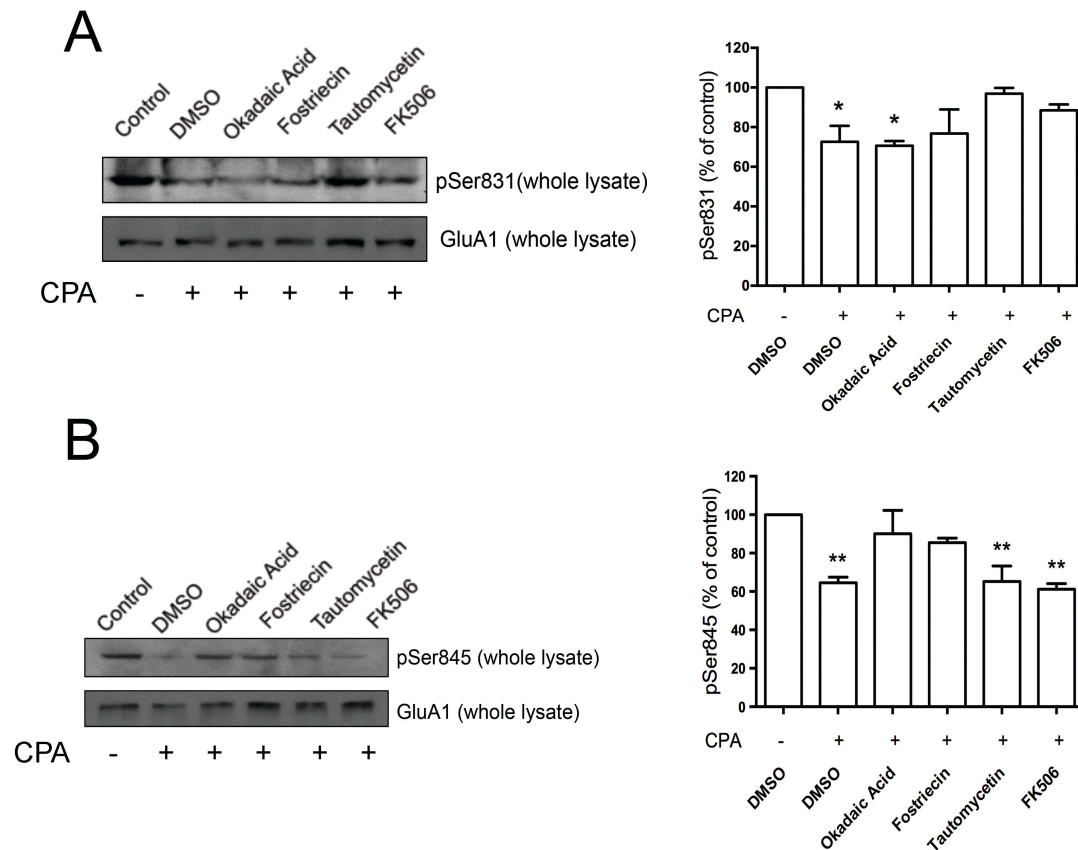


Figure 2.2. Dephosphorylation of GluA1 at Ser831 and Ser845 is mediated by A1R-induced protein phosphatases 1, 2A and 2B activity. Western blots of hippocampal lysates treated with protein phosphatase inhibitors. (A) *left*: Representative whole lysate blots of hippocampal lysates treated with okadaic acid (20nM), fostriecin (20nM), tautomycin (20nM), or FK506 (50nM) for 1h prior to additional 30min CPA treatment. Phosphorylated Ser831 (pSer831) of GluA1 was reduced after CPA treatment, and tautomycin significantly reduced this effect. GluA1 expression was not significantly different between treatments, indicating no reduction in whole cell GluA1 levels.. *right*: Summary bar graph of results for pSer831 levels in right blot. (B) *left*: Representative blots showing that CPA treatment also significantly reduced levels of GluA1-pSer845, which was prevented by okadaic acid and fostriecin treatment. Tautomycin and FK506 did not prevent this CPA-induced reduction in pSer845. *right*: summary bar graphs show that pSer845 was significantly reduced by CPA

treatment alone, with tautomycetin, and with FK506, but not with okadaic acid and fostriecin, indicating that PP2A mediates CPA-induced reduction of pSer845. N=3 independent blots/experiments. Average values are mean \pm SEM, Significance values: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

2.4.3 Synaptic depression and APSD in area CA1 are reduced by protein phosphatase inhibition.

To examine the hypothesis that protein phosphatase inhibition prevents CPA-induced synaptic depression and reduced APSD, described previously (Chen et al., 2014), we performed fEPSP recordings in rat hippocampal slices. CA1 region fEPSP recordings were evoked from hippocampal slices pre-treated with protein phosphatase inhibitors (see above) for a minimum of 1h prior to recording. APSD was induced using a 30min CPA treatment (500nM) followed by a 1h washout period. Control (DMSO) slices showed significant synaptic depression (~80%, n=10 slices, all from different rats) with CPA treatment, which did not recover to baseline levels during washout, indicating APSD (Figure 2.3A,B). Hippocampal slices that were treated with the protein phosphatase inhibitors okadaic acid (20nM, n=8), fostriecin (20nM, n=9), tautomycetin (20nM, n=7) and FK506 (50nM, n=9) showed reduced synaptic depression during CPA treatment, accompanied by reduced APSD in washout (Figure 2.3).

Synaptic depression after CPA treatment (Figure 2.3B,C) was significantly reduced by pre-treatment with the protein phosphatase inhibitors, with okadaic acid most able to prevent synaptic depression and FK506 preventing the least synaptic depression. During washout, the amount of fEPSP recovery was compared between treatment groups. Compared to control (DMSO), okadaic acid, fostriecin, FK506, and tautomycetin improved the amount of fEPSP recovery in washout (Figure 2.3D), with each recovering by about 30% during the 1h

washout period, similarly to control. Although there was less synaptic depression and APSD in slices treated with phosphatase inhibitors, APSD was still observed in protein phosphatase inhibitor-treated slices, indicating that protein phosphatases, although important, are not the only proteins involved in APSD.

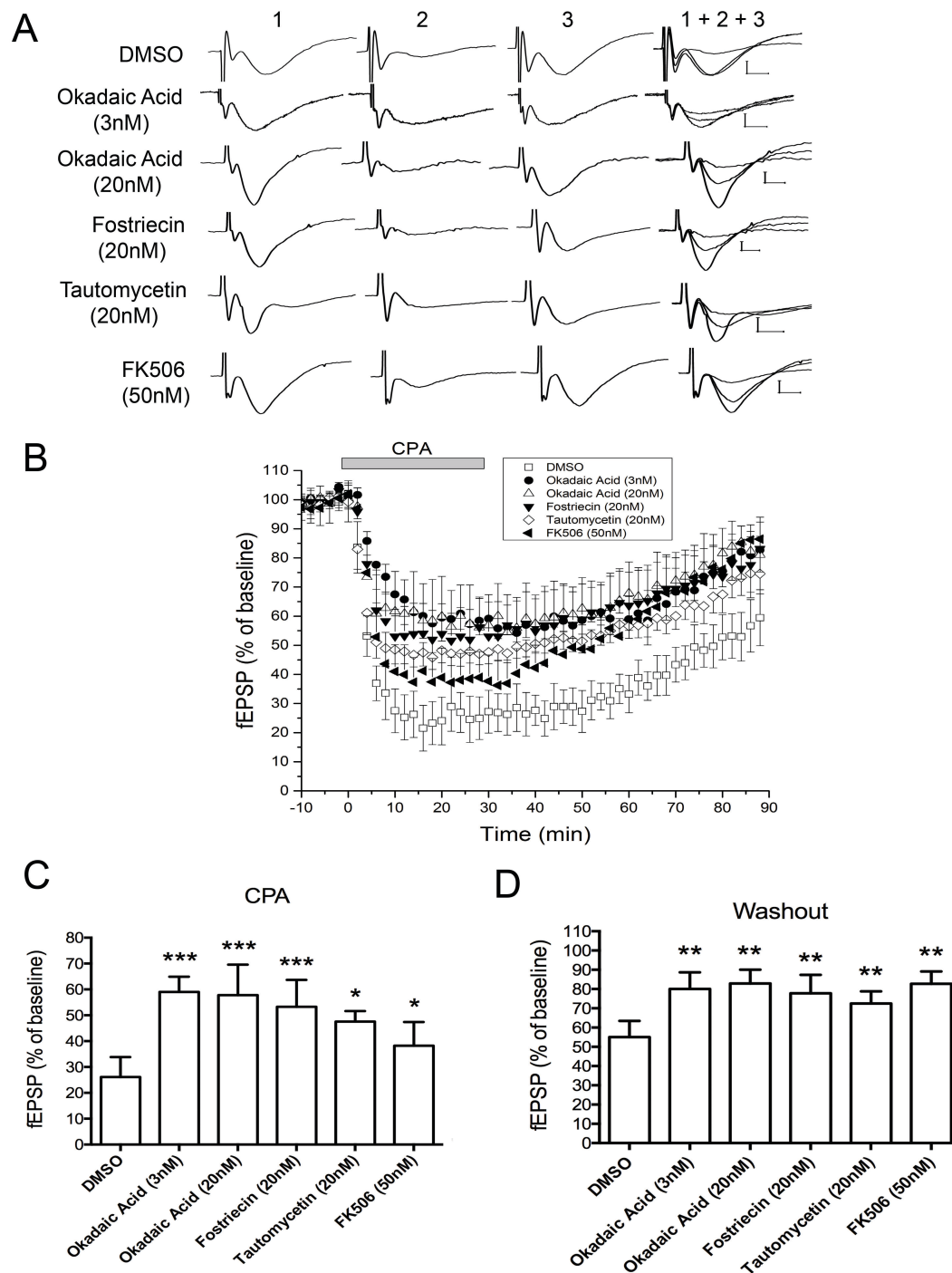


Figure 2.3 Synaptic depression in area CA1 is reduced by protein phosphatase 1, 2A, and 2B inhibition. (A) Representative fEPSP traces for each treatment showing a sample trace at the end (final 5min) of the 20min baseline recording (1), at the end of 30min CPA treatment (2), at the end of 1h washout period (3), and an overlay of the three representative traces (1+2+3). Each representative trace is an average of the final 10 sweeps (5min) of each

of the three parts of the experiment (baseline, CPA, and washout). All recordings were normalized to their own baseline value (100%). (B) Time-course plot of average fEPSP slopes normalized to baseline (100%). (C) Summary bar graph showing fEPSPs as a percentage of the baseline recording level at the end of a 30min CPA treatment. Hippocampal slices treated with CPA and no drug inhibitors (DMSO) showed approximately 75% synaptic depression. Okadaic acid, fostriecin, tautomycetin, and FK506 incubation for 1h prior to the experiment significantly reduced the amount of synaptic depression caused by CPA. (D) Summary bar graph of the mean fEPSP slope at the end of the 1h washout period. DMSO slices recovered by about 20%, with fEPSPs recovering to about 50% of baseline. On the other hand, the fEPSP slopes for slices treated with protein phosphatase inhibitors was significantly higher at the end of the washout period. $n=7-10$ slices were recorded for each treatment group from different rats. Graphed values are shown as mean \pm SEM. Significance values: $*$ = $p < 0.05$, $**$ = $p < 0.01$, $***$ = $p < 0.001$.

2.4.4 Protein phosphatase inhibitor treatment before hypoxia altered synaptic depression during hypoxia and post-hypoxic potentiation in normoxic washout

To test the functional involvement of PP1, PP2A and PP2B in hypoxia-induced synaptic depression and subsequent synaptic recovery during reperfusion, we subjected hippocampal slices pre-treated with okadaic acid, fostriecin, tautomycetin or FK506 (same concentrations as above) to a 20min hypoxic insult followed by a 45min normoxic reperfusion. Hypoxia was induced by bubbling aCSF with 95% N_2 /5% CO_2 for a minimum of 20min prior to the start of the experiment. This experimental protocol was used to mimic an ischemia/reperfusion (I/R) injury, and to examine the role of protein phosphatases in mediating the cellular response to I/R injury. Hypoxic insult induced rapid synaptic depression in all hippocampal slices, with no significant difference between control and okadaic acid, fostriecin, tautomycetin, or

FK506 treated slices (Figure 2.4B,C). In normoxic washout, control (DMSO) slices recovered to baseline quickly and then continued to potentiate to levels approximately 160% of baseline (Figure 2.4B,D). This post-hypoxic potentiation was seen to differing degrees in the protein phosphatase inhibitor-treated slices, only FK506-treated slices significantly reduced post-hypoxia potentiation much closer to baseline levels (~110%). These results indicate that A1R-mediated protein phosphatase activation is only one of many cellular response mechanisms that are activated in hypoxia. In normoxic reperfusion, post-hypoxia potentiation occurred, which was significantly reduced by FK506 treatment, indicating a differential role for these phosphatases during hypoxia/reperfusion injury.

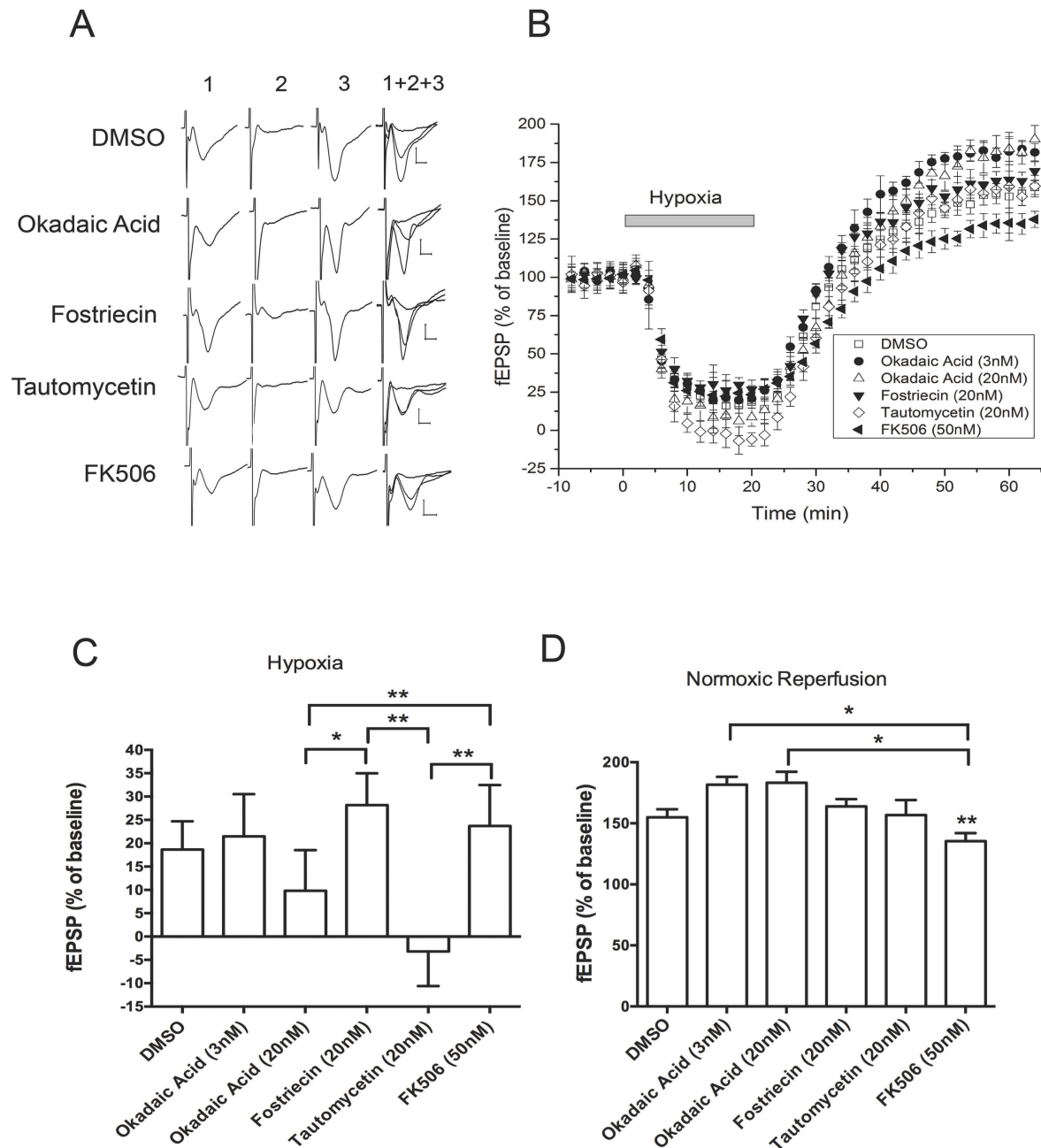


Figure 2.4 Protein phosphatase inhibitor treatment did not significantly alter hypoxia-induced synaptic depression, but FK506 prevented post-hypoxia potentiation in normoxic reperfusion. (A) Representative fEPSP traces shown as an average of the final 10 traces (5min) of each treatment period: 1. baseline recording, 2. hypoxia, 3. normoxic reperfusion, and 1+2+3 shows an overlay of the three representative traces. (B) Time-course plot of fEPSP experimental data showing mean fEPSP slopes for each recording plotted as a percentage of baseline values (100%), with each trace showing the average of normalized

fEPSP data for each independent n value. (C) Summary bar graph showing average fEPSP data for the final 10 sweeps (5min) of hypoxia. Compared to control, there were no significant changes in fEPSP slope, but tautomycetin (20nM) and okadaic acid (20nM) treatment showed significantly less synaptic depression than fostriecin (20nM) and FK506 (50nM) treatment. (D) Summary bar graph showing the final 5min of a 45min normoxic washout period. Control (DMSO) slices showed significant synaptic potentiation following hypoxia, with slices potentiating to about 160% of baseline (100%). FK506 treatment significantly reduced this post-hypoxia potentiation, with slices potentiating to only about 135% of baseline values. N = 7-9 separate experiments for each treatment. Data is shown as mean \pm SEM. Significance values: * = $p < 0.05$, ** = $p < 0.01$.

2.4.5 Post-hypoxia reperfusion increased surface GluA1, but not GluA2, which is removed after A2A receptor inhibition.

We previously showed that a 20min hypoxic insult to hippocampal slices induces reduced AMPAR and A1R surface expression, and increases A2AR surface expression (Chen et al., 2014). We hypothesized that following hypoxia, A1R internalization corresponds to A1R desensitization, and that excitatory A2AR activation was contributing to the post-hypoxic potentiation seen during normoxic washout in the above fEPSP experiment (Figure 2.4). Following 20min of hypoxia, both GluA1 and GluA2 surface expression is significantly reduced compared to control (Figure 2.5A). Whole cell levels of GluA1 and GluA2 did not change, indicating that there is no significant AMPAR degradation inside the cell.

To test whether A2ARs induce increased AMPAR surface expression during normoxic washout, hippocampal slices were treated with SCH442416 (5nM) to inhibit A2AR activation 1h prior to inducing hypoxic insult. Slices were then subjected to 20min hypoxia followed by 45min normoxia and then immediately biotinylated. Western blot data from

these experiments show that after the 45min normoxic washout period, GluA1 surface expression, but not GluA2 expression, was significantly increased compared to control (Figure 2.5D-F). Treatment with SCH442416 prevented increased GluA1 surface expression, indicating that A2ARs induce the increased GluA1 surface expression seen in hypoxia alone (Figure 2.5D,E, middle lane). Normoxic washout did not increase GluA2 surface expression after hypoxia, and SCH442416 did not alter surface levels of GluA2 compared to hypoxia and subsequent normoxia alone (Figure 2.5D,F), indicating that A2ARs did not induce increased GluA2 surface levels in post-hypoxic conditions. Taken together, these data indicate differential regulation of GluA1 and GluA2 surface expression after a hypoxic insult, with A2AR activation during normoxic washout inducing increased GluA1, but not GluA2 surface expression.

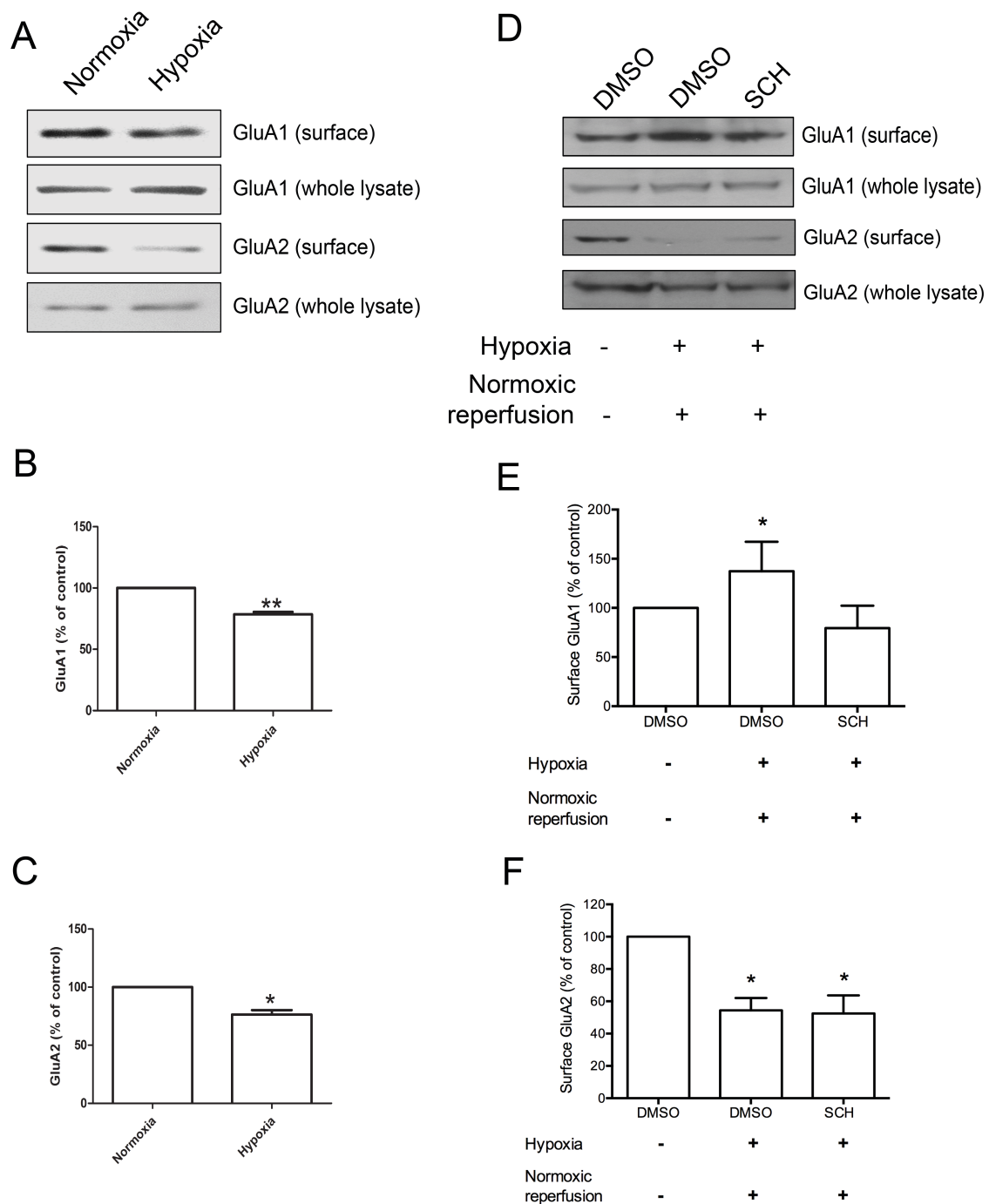


Figure 2.5 Hypoxia induced GluA1 and GluA2 internalization, whereas after normoxic washout, A2ARs induce increased GluA1, but not GluA2, surface expression. (A) Representative biotinylated Western blots of hippocampal slices subjected to 20min hypoxia and then immediately biotinylated. Both GluA1 and GluA2 surface expression was decreased after hypoxia. (B) Summary bar graph of GluA1 surface expression with and without hypoxia

shown in A (top two blots). Hypoxia induces significantly reduced GluA1 surface expression compared to control. (C) Summary bar graph showing surface GluA2 levels with and without a 20min hypoxic insult showing reduced surface GluA2 after hypoxia. (D) Representative blots showing surface expression of GluA1 and GluA2 in hippocampal slices subjected to 20min hypoxia followed by 45min normoxic washout. Blots show increased GluA1 after the washout period which was prevented with the treatment of SCH442416 (5nM), an A2AR-selective inhibitor. GluA2 levels were reduced after the normoxic washout with or without SCH442416 treatment. (E) Summary bar graph of surface GluA1 expression after normoxic washout. GluA1 levels were significantly increased after normoxia, and this increase was prevented by SCH442416, with GluA1 surface levels comparable to control slices. (F) Summary bar graph showing surface GluA2 levels. SCH442416 did not alter the hypoxia-normoxic washout decrease in surface GluA2. N=3 independent blots with at least 2 rats per experiment. Values are shown as mean \pm SEM. Significance values: * = $p < 0.05$, ** = $p < 0.01$. Figure 2.5 A, B, and C were gathered with the help of Zhicheng Chen, who performed Western blot experiments in these figures.

2.4.6 Following normoxic washout after hypoxia, there is increased pSer831 and pSer845 in hippocampal slices compared to time control, and A2AR inhibition prevented hypoxia-reperfusion-induced increase in pSer831 and pSer845.

A2ARs are known to induce increased PKA activity, which is known to phosphorylate GluA1 at Ser845 and increase GluA1 surface expression (Dias et al., 2012). We hypothesized that A2AR blockade prior to 20min hypoxia and 45min normoxic washout would prevent increased GluA1 phosphorylation. After the 45min washout period, both pSer831 and pSer845 increased in biotinylated and whole lysate blots (Figure 2.6). Blots for pSer831 show that hypoxia followed by normoxic washout induced significantly increased pSer831 to about

140% of baseline levels (Figure 2.6A,B). Pre-incubation of slices with SCH42416 (5nM, A2AR antagonist) reduced the increase of pSer831 to about 120% of baseline, which was not significantly different from control or hypoxia alone, but reduced compared to hypoxia alone. Levels of pSer845 was significantly increased to about 120% above control after normoxic washout, which was prevented with SCH442416 treatment, where pSer845 levels returned to control levels (Figure 2.6C,D). These results indicate that A2ARs are involved in hypoxic reperfusion-induced phosphorylation of GluA1 at Ser831 and Ser845, which corresponds to increased A2AR-mediated GluA1 surface expression (Figure 2.5).

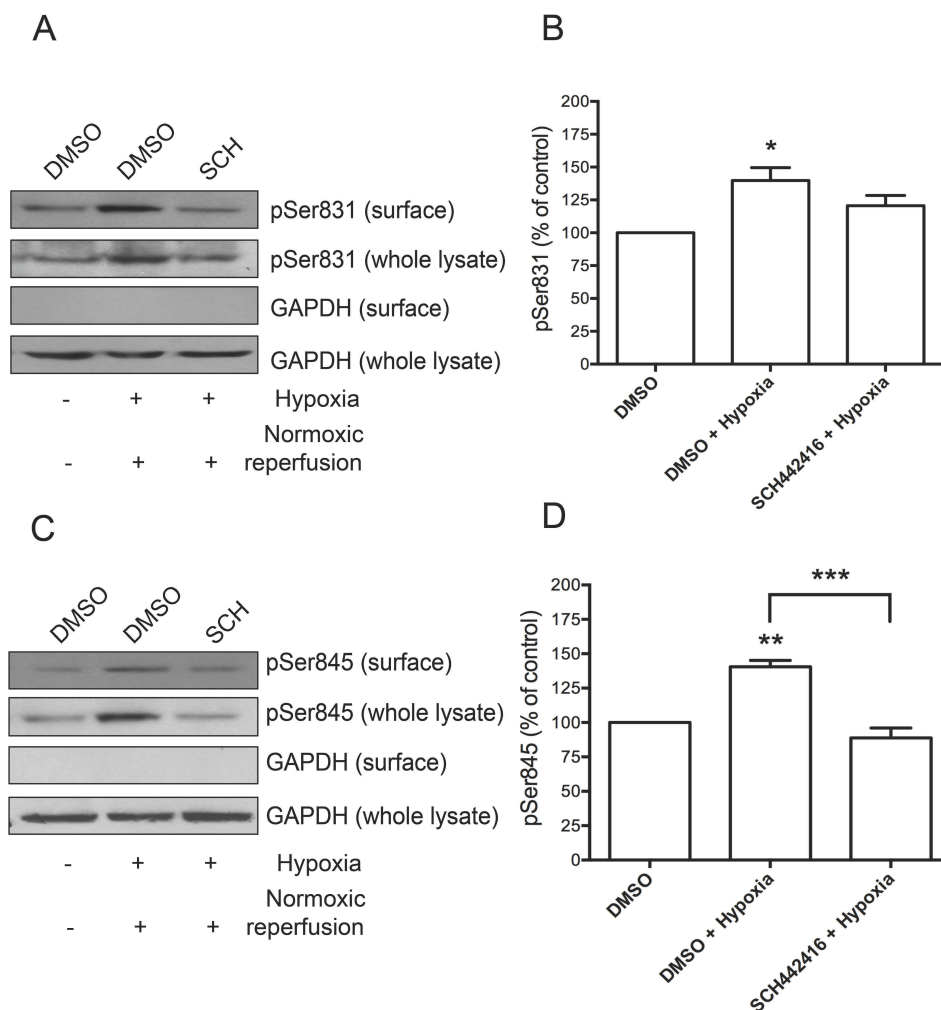


Figure 2.6 After hypoxia and normoxic washout of hippocampal slices, pSer831 and pSer845 are increased, and SCH442416 prevented this increase. (A) Representative blots showing that compared to time-control slices (DMSO, far left lane), treatment with 20min hypoxia followed by 45min normoxic reperfusion caused significantly increased levels of cellular pSer831 (DMSO, center lane). Slices that were treated with SCH442416 (5nM) 1h prior to the hypoxia-reperfusion experiment (right lane) prevented the hypoxia-reperfusion-induced increase in pSer831. (B) Summary bar graph showing average data for blots shown in A. Hypoxia-reperfusion induced significantly increased pSer831 compared to time-control (100%). Slices treated with SCH442415 before hypoxia-reperfusion showed increased pSer831 levels compared to control, but not significantly (i.e. $P > 0.05$). (C) Representative

blots of a membrane used for experiment in A that were stripped and re-probed for pSer845 levels. Hypoxia-reperfusion-treated slices showed significantly higher pSer845 levels compared to time control, and SCH442416-treated slices showed no increase in pSer845 levels after hypoxia and reperfusion alone. (D) Summary bar graph showing average data from all blots analyzed for this experiment. Graphed values are shown as mean \pm SEM, with n=4 independent experiments performed. Significance values: *= p<0.05, **= p<0.01, ***= p<0.005.

2.4.7 Hippocampal slices treated with SCH442416 with or without protein phosphatase inhibitors prevented the induction of post-hypoxia potentiation.

Following the discovery that A2ARs mediate post-hypoxic increases in surface GluA1 and pSer831/pSer845 (Figure 2.5, 2.6), we performed fEPSP experiments to test whether SCH442416 (5nM) treatment prevents adenosine-induced post-hypoxic potentiation, and whether A2AR blockade modulates post-hypoxia potentiation during normoxic washout. In hypoxia, there were no significant differences in hypoxia-induced synaptic depression between treatment groups, consistent with the previously shown increase in extracellular adenosine in hypoxia (Dale et al., 2000) (Figure 2.7C). Following 20min hypoxia, slices were then reperused with normoxic solution for 45min. At the end of the washout period, control slices showed significant (~160%) fEPSP potentiation. On the other hand, slices that were preincubated in SCH442416 alone or with okadaic acid (20nM), fostriecin (20nM), tautomycin (20nM) or FK506 (50nM) showed that SCH442416 prevented post-hypoxia potentiation (Figure 2.7D). This result indicates that post-hypoxia potentiation is mediated through A2AR activation, which has been shown to induce PKA activation, which in turn phosphorylates GluA1 causing increased GluA1 surface expression (Dias et al., 2012), which is consistent with experiments above (Figure 2.5, 2.6).

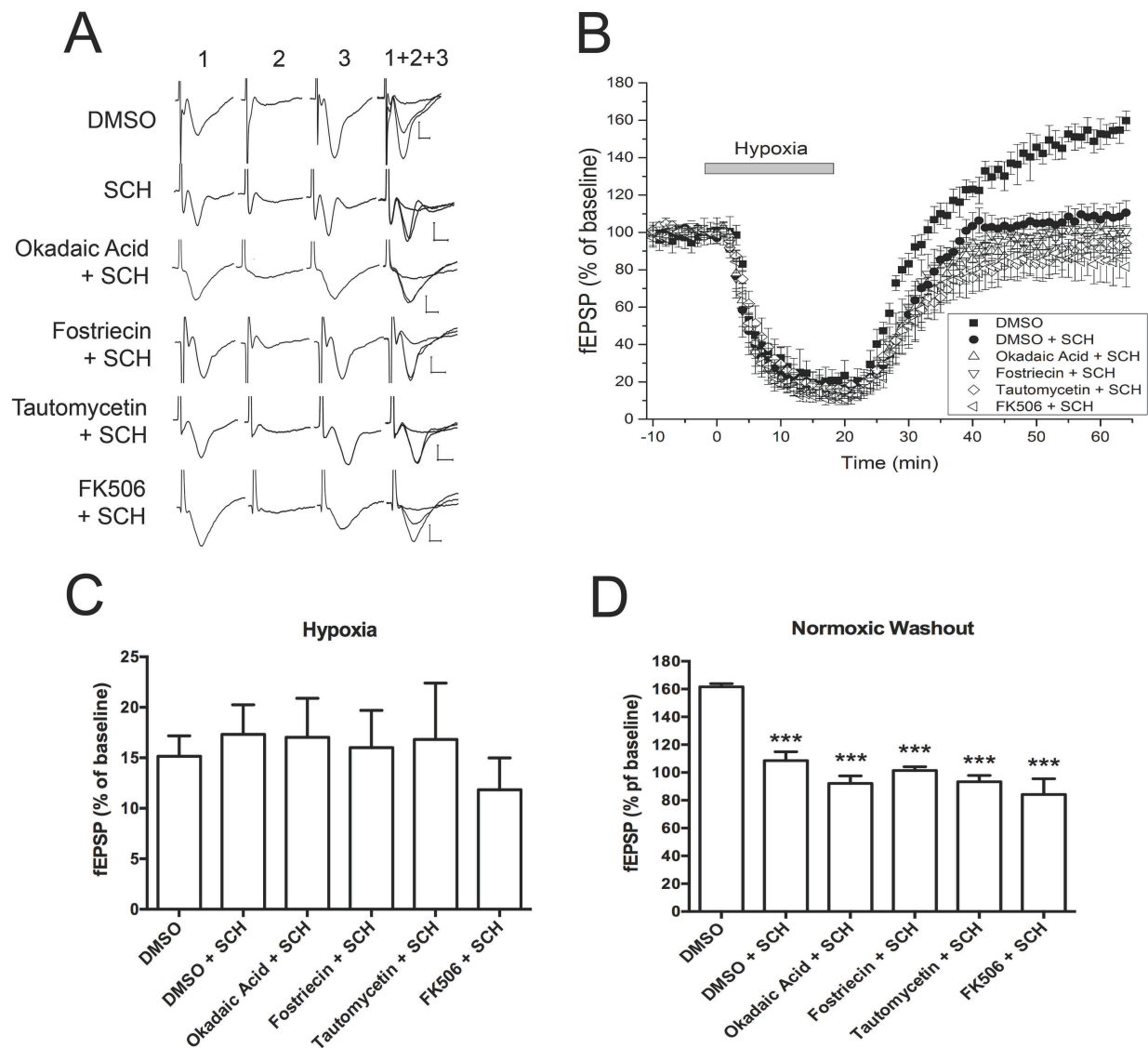


Figure 2.7. A2AR inhibition abolished Adenosine-induced post-hypoxia potentiation (APHP) in CA1 hippocampal slices following 20min hypoxia and normoxic reperfusion.

(A) Representative fEPSP at experimental time points of the final 10 traces (5min) prior to the end of a treatment period (20min baseline, 20min hypoxia, 45min normoxic reperfusion). fEPSP traces in column 1 (far left) show representative fEPSP traces for the final 5min of the baseline recording period. Column 2 (second from the left) shows traces at the end of hypoxia treatment, and column 3 (third from the left) shows traces at the end of the 45min normoxic reperfusion period. Finally, column 4 (titled 1+2+3, right side) shows an overlay of

each of the 3 traces for each treatment group. Scale bars are 0.25mV in the Y-axis and 10ms in the X-axis. (B) fEPSP time-course plot of the average normalized fEPSP slopes for each drug incubation. (C) Summary bar graph showing the average of the final 10 sweeps of each treatment group at the end of the 20min hypoxia period. During hypoxia in the presence of SCH442416 with various protein phosphatase inhibitors, there is no significant difference between any of the treatment groups, indicating that A2ARs do not affect hypoxia-induced synaptic depression. (D) Summary bar graph of fEPSPs after 45min normoxic washout. In normoxic reperfusion, the addition of SCH442416 (5nM) to hippocampal slices abolished post-hypoxia potentiation. (E) Bar graph showing the comparison between the difference of normalized fEPSP slope without (from Figure 2.4) vs. with SCH442416 in the final 5min of the hypoxia treatment. Results show that there were no significant differences between the values with or without SCH442415 treatment. (F) Bar graph showing a similar comparison as in E, except calculated using values taken at the end of the normoxic washout period. SCH442415 reduced fEPSP values the most in control (DMSO) slices, whereas FK506-treated slices showed significantly less of a difference with or without SCH442416 treatment. N = 7-9 separate experiments for each treatment. Values are shown as mean \pm SEM. Significance values: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$. Data was acquired with the assistance of Dr. Zhi Ming: Dr. Ming performed fEPSP recordings, and experimental design, collection of preliminary data, and data analysis were performed by myself.

2.4.8 Protein phosphatase inhibitor pre-incubation reduced neuronal death in pyramidal CA3-CA1, with significant neuronal protection in CA1 of hippocampal slices after 20min hypoxic insult.

To test the neuroprotective potential of these protein phosphatase inhibitor drugs used in these experiments, propidium iodide (PI) fluorescent staining was used, which selectively

stains dead cells with disrupted plasma membranes (Pugliese et al., 2009). The PP2B inhibitor FK506 has been shown to be neuroprotective in various *in vitro* and *in vivo* preparations (Sharkey and Butcher, 1994; Butcher et al., 1997; Bochen et al., 1999; Uchino et al., 2002), and due to similar effects of PP1 and PP2A inhibitors found in the above figures, we hypothesized that these treatments would show neuroprotection in hippocampal slices subjected to a prolonged 20min hypoxic insult.

Hippocampal slices were pre-incubated in the protein phosphatase inhibitors okadaic acid (3nM), fostriecin (20nM), tautomycin (20nM), and FK506 (50nM) for 1h followed by a 20min hypoxic insult. It is important to note that okadaic acid has been shown to cause neuronal death alone (Nuydens et al., 1998), so a lower concentration (3nM compared to 20nM) of okadaic acid was used in this experiment to reduce the chance of inducing okadaic acid-induced neuronal death. Following a 3h incubation and PI staining in the final hour of incubation, slices were fixed in paraformaldehyde and imaged using a Zeiss LSM700 laser scanning confocal microscope. Resultant images show that hypoxia control (DMSO) caused significant neuronal death in CA1 compared to time control slices (Figure 2.8A,G). All four protein phosphatase inhibitor treatments significantly reduced neuronal death after hypoxia, with neurodegeneration seen in fostriecin and FK506-treated slices were significantly less than DMSO (Figure 2.8D,E). These results indicate that protein phosphatase inhibitors afford neuroprotection to hippocampal slices subjected to a prolonged hypoxic insult.

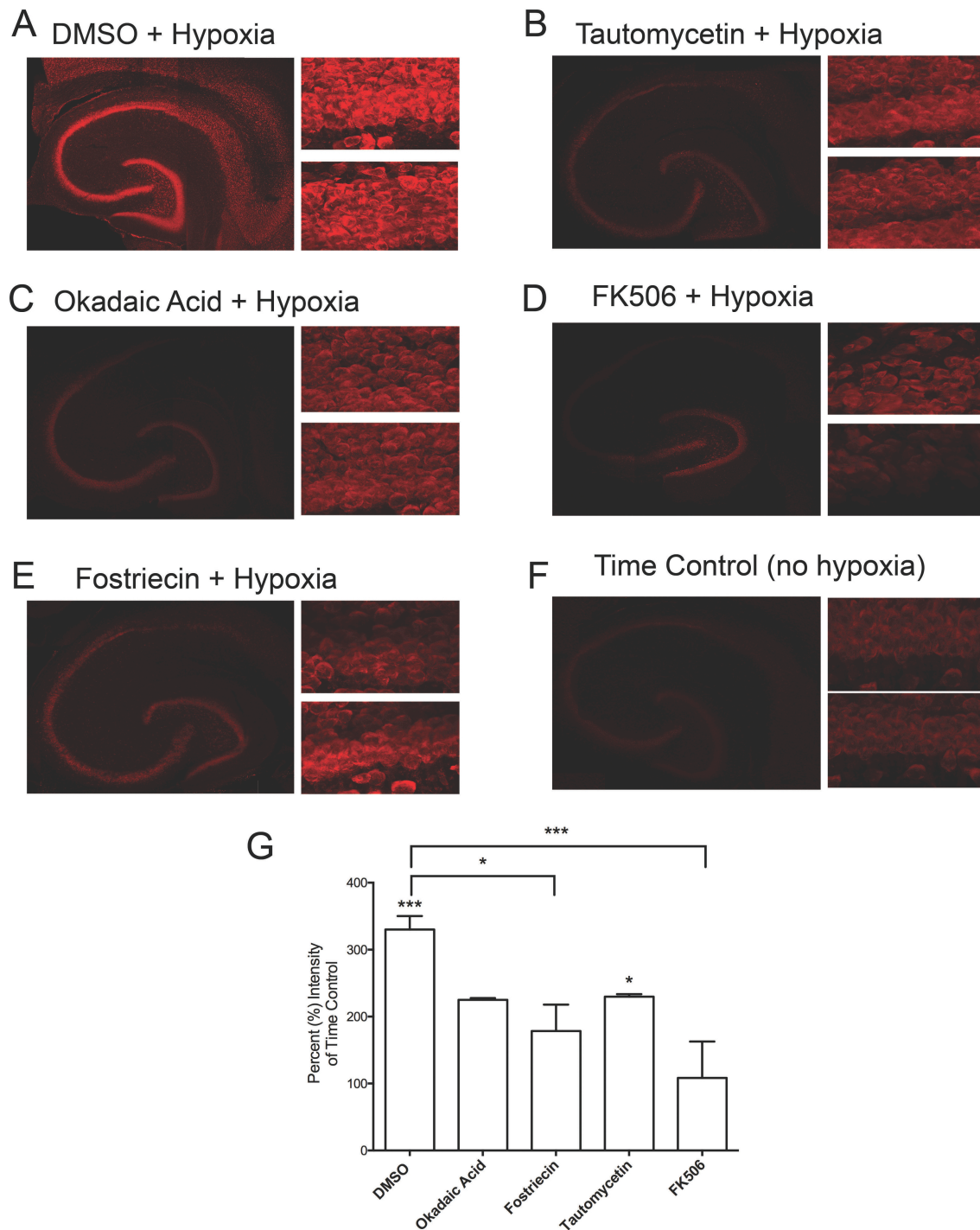


Figure 2.8 Protein phosphatase inhibitor treatment reduced neurodegeneration in hippocampal slices following 20min hypoxia. Propidium iodide-labeled hippocampal slice montages show representative slices for each treatment group (A-F). (A) Control (DMSO) hypoxia slices showed high levels of neuronal death in the dentate gyrus and CA3-CA1 (left panel). The right panel shows two representative combined Z-stack images of CA1 showing

significant neuronal death. (B) Slices treated with the PP1 inhibitor tautomycin (20nM) showed reduced neuronal death in all hippocampal regions (left panel) and Z-stack images of CA1 (right two panels) show reduced neuronal death. (C) Representative slice treated with Okadaic Acid (3nM) showing both the whole hippocampus (left) and two Z-stack images (right) (D) Representative images of whole hippocampal slices (left) treated with FK506 (50nM) prior to hypoxia, with two Z-stack images (right) on CA1. (E) Representative images of foscarnin-treated hippocampal slice (20nM), panels same as above. (F) Representative time control slice images showing the least neurodegeneration. (G) Summary bar graph showing mean densitometry values normalized to the time control values (100%). Representative Z-stack images are shown as a maximum intensity image of 20 μ m inside 400 μ m thick hippocampal slices. n = 4 independent experiments, using 5 rats per experiment.. Values are shown as mean \pm SEM. Significance values: * = $p < 0.05$, *** = $p < 0.005$.

2.5 Discussion

In this study, we investigated the role of protein phosphatases 1, 2A and 2B in adenosine-mediated synaptic depression in hypoxia/reperfusion injury in rat hippocampal slices, and their role in regulating AMPA receptor surface expression. We show that these three protein phosphatases mediate A1R-induced GluA1 endocytosis by reducing the phosphorylation of Ser831 and Ser845 GluA1 C-terminal residues. PP1, PP2A and PP2B inhibition reduced CPA-induced GluA1 internalization, but only PP2A inhibition prevented CPA-induced GluA2 endocytosis. Accordingly, the levels of GluA1 pSer831 and pSer845 were reduced by CPA, and this reduction in pSer831 and pSer845 was prevented by treatment of hippocampal slices with PP1, PP2A and PP2B inhibitors. Along with reducing AMPAR surface

expression, prolonged A1R activation induced APSD (Chen et al., 2014), which was reduced by pre-treating hippocampal slices in PP1, PP2A or PP2B inhibitors.

After a 20min hypoxic insult, both control and protein phosphatase inhibitor-treated hippocampal slices showed fEPSP recovery in normoxic reperfusion above baseline, which we have termed *adenosine-induced post-hypoxic potentiation* (APHP) (Figure 2.4). Treatment with SCH442415 (5nM), an A2AR antagonist, prevented APHP (Figure 2.7), indicating that A2AR activation following oxygen reperfusion after hypoxia mediates APHP, potentially enhanced by A1R desensitization after hypoxia (Chen et al., 2014). Based on these data we suggest that hypoxia-induced A1R activation influences A2AR activity in reperfusion.

Slices subjected to 20min hypoxia followed by 45min normoxic reperfusion/washout showed increased surface GluA1, but not GluA2, levels during reperfusion-induced APHP, which was prevented by pre-treatment with SCH442416 (Figure 2.5). Increased GluA1 surface expression after washout was accompanied by increased pSer831 and pSer845 levels, which was also prevented with SCH442416 treatment (Figure 2.6). Finally we found that, protein phosphatase inhibitor treatments prior to a 20min hypoxic insult were shown to be neuroprotective in hippocampal slices. Our study reports a novel mechanism mediating a functional interaction between A1Rs and GluA1, where protein phosphatases induced GluA1 endocytosis following A1R activation.

PP1, PP2A and PP2B have been shown to contribute to the modulation of excitatory neurotransmission and learning and memory processes throughout the brain through a multitude of intracellular mechanisms (Mulkey et al., 1993; Lin et al., 2000; Belmeguenai and Hansel, 2005). For example, PP1 activity has been shown to mediate the induction of LTP and LTD (Allen et al., 2000; Li and Pan, 2013), and has been shown to directly regulate AMPA receptors in the brain (Yan et al., 1999). In addition to previously described A1R-

induced increase in active PP2A in the postsynaptic density (Brust et al., 2006), PP2A has also been shown to mediate multiple functions in the brain which also includes direct modulation of AMPARs (Launey et al., 2004). It has also been shown that the levels of the regulatory B subunit of PP2A is decreased after focal ischemia, which indicates increased PP2A activity (Koh, 2011). Finally, PP2B, a Ca^{2+} -dependent protein phosphatase, has been implicated in the induction of neurotoxicity in ischemia (Ankarcrona et al., 1996; Asai et al., 1999), whereas inhibition of PP2B with FK506 is an effective neuroprotective treatment (Sharkey and Butcher, 1994; Butcher et al., 1997; Bochelen et al., 1999; Uchino et al., 2002). In this study, we add to the wide list of functions performed by these three ubiquitous protein phosphatases.

We recently showed that p38 MAPK and JNK mediate A1R-induced GluA2 endocytosis, with a smaller effect on GluA1 endocytosis, and PP2A is involved in both GluA1 and GluA2 endocytosis (Chen et al., 2014). The results of this chapter show a novel mechanism by which adenosine modulates synaptic activity in the hippocampus in hypoxic injury through the activation of PP1, PP2A and PP2B, which induce GluA1 internalization through reduction in the phosphorylation of Ser831 and Ser845. Previous reports have described the involvement of these three protein phosphatases in various ischemia-induced cellular mechanisms in the brain (Rundén et al., 1998; Cid et al., 2007). For example, PP1, PP2A, and PP2B were shown to play a role in estrogen-mediated neuroprotection in ischemia, where inhibition of these protein phosphatases reduced estrogen-mediated neuroprotection after *transient middle cerebral artery occlusion* (tMCAO) *in vivo* stroke model, supporting a neurodegenerative effect of protein phosphatase 1, 2A and 2B inhibition (Walsh et al., 1997). In contrast, our results indicate a neuroprotective action of okadaic acid, fostriecin, tautomycin and FK506, which supports a neuroprotective effect of the inhibition of PP1, PP2A and PP2B (Figure 2.8). Although these results appear to contradict, it is important to

consider that these three protein phosphatases are ubiquitous and have a wide range of substrates and activators, and these two studies used two different models, one being *in vivo* and this study being *in vitro*.

It has been recently shown that a 10min oxygen-glucose deprivation (OGD) insult to hippocampal slices induced a reduction in excitatory postsynaptic currents (EPSCs) followed by increased recovery above baseline levels, which has been termed ischemia-induced long-term potentiation (i-LTP) (Dias et al., 2013). The use of 20min hypoxia in the current study mimicked the effect seen by Dias et al. (2013), showing significant synaptic depression in hypoxia followed by fEPSP potentiation during normoxic reperfusion. Both i-LTP and APHP involve A2AR activation and increased CP-AMPA activity, and potentiation can be reduced by either A2AR inhibition or CP-AMPA inhibition (Dias et al., 2013). These data indicate that GluA1-containing AMPARs and A2ARs are key mediators of reperfusion injury to neurons by inducing increased Ca^{2+} permeability, thus increasing the chance of excitotoxicity. GluA1 and GluA2 are the most abundantly expressed AMPAR subunits in the brain and the presence or absence of GluA2 in a functional tetramer receptor confer Ca^{2+} -permeability to the receptor (Rogers et al., 1991). Increase CP-AMPA surface expression is a known mechanism for the induction of LTP (Szabo et al., 2012), which shows similarities to i-LTP, except that CP-AMPA expression is transient in LTP, whereas it is more prolonged in i-LTP (Szabo et al., 2012), which may explain why CP-AMPA expression is not neurotoxic in LTP induction.

Finally, propidium iodide was used to label hippocampal slices for neurodegeneration, and protein phosphatase 1, 2A and 2B inhibitor drug pre-treatments reduced the amount of neurodegeneration caused by a 20min hypoxic insult. By inhibiting PP1, PP2A, or PP2B, A1R-mediated AMPAR internalization is reduced (Esteban et al., 2003; Lu and Roche, 2012), which would contribute to increased neuronal excitability in hypoxia. In particular,

since p38 MAPK and JNK also mediate A1R-induced GluA2 internalization (Chen et al., 2014), there may be increased GluA1-containing AMPAR surface expression with reduced GluA2-containing AMPAR surface expression. Increased GluA1 surface expression through reduced internalization and increased pSer831 and pSer845 has been shown to increase neuronal excitation and increase Ca^{2+} permeability (Liu and Zukin, 2007). Additionally, increased GluA2 internalization has been shown to induce pro-apoptotic intracellular signals (Gorter et al., 1997). Interestingly, fEPSP experiments show that protein phosphatase inhibition did not prevent APHP following 20min hypoxia, except in the case of FK506 (Figure 2.7). This is important because APHP may be associated with excitotoxicity (Dias et al., 2012). To elucidate the mechanism of protein phosphatase inhibitor-induced neuroprotection while not preventing APHP in normoxic washout, further research is required to test the involvement of other receptors (eg. NMDARs) and intracellular kinases or phosphatases in these effects.

Chapter 3: Regulation of extracellular adenosine levels by the interaction of adenosine A1 receptors, casein kinase 2, and equilibrative nucleoside transporters in hypoxia and focal cortical ischemia in rat hippocampal slices

3.1 Summary

Dynamic regulation of the levels of extracellular adenosine occurs in both physiologically- and pathophysiologically-induced cellular reactions to various normal and noxious stimuli in the hippocampus. In hypoxia or ischemia, adenosine levels rapidly increase up to 100 times above normal levels, and mediate rapid synaptic depression through activation of high-affinity adenosine A1 receptors (A1Rs), which are responsible for synaptic depression. Additionally, equilibrative nucleoside transporters (ENTs), which are abundantly expressed in the hippocampus, allow passive transport of adenosine across cell plasma membranes. Due to the passive transport of ENT channels, a major mechanism of the regulation of adenosine transport through ENTs is by the modulation of ENT surface expression. In this chapter, casein kinase 2 (CK2) will be investigated as a potential modulator of adenosine-induced neuronal responses. CK2, a ubiquitous serine/threonine kinase, has been implicated in a wide array of intracellular signaling activity in the hippocampus. The ENT1 channel subtype contains intracellular amino acid consensus sequences that are targeted by CK2, indicating that CK2 may play a role in ENT1 regulation. Paired pulse fEPSP experiments in CA1 of rat hippocampal slices were performed to assess the effect of CK2 and ENT inhibitors on adenosine tone. Results indicate that there was increased adenosine A1R activation following CK2 or ENT inhibitor treatments. Additionally, fEPSPs with a 20min hypoxic insult were performed to assess the effect of CK2 and ENT inhibitors on the electrophysiological response to hypoxia. Inhibition of CK2 or ENT1 and ENT2 induced increased extracellular adenosine tone, and also reduced hypoxia-induced synaptic depression and prevented *adenosine-induced post-hypoxia potentiation* (APHP). Additionally, CK2 inhibitor treatment was shown to be neuroprotective after a 20min hypoxic insult. These results indicate that CK2 regulates ENT1 following its activation by A1Rs. Finally, this chapter examines the paired-pulse ratio before and after A1R inhibition in hippocampal slices taken from rats 48

hours after pial vessel disruption (PVD) small vessel cortical stroke surgery. PVD animals showed significantly different effects after A1R inhibition, suggesting that PVD induced changes in adenosine signaling in the hippocampus.. These data illustrate the importance of the regulation of extracellular adenosine, and suggest CK2 as a potential therapeutic target for future studies.

3.2 Introduction

Equilibrative nucleoside transporters (ENTs) are a major mediator of the movement of adenosine and other nucleosides such as guanosine across cell membranes, and they are dynamically regulated (Anderson et al., 1999; Baldwin et al., 2004). In hypoxia and cerebral ischemia, extracellular adenosine increases appreciably (Dale et al., 2000), with two potential sources being adenosine extruded from ischemic cells and from extracellular adenosine metabolism by ectonucleotidases (Latini et al., 1996b). It has been shown that the high-affinity adenosine A1 receptor (A1R) is highly activated in the hippocampus following increased extracellular adenosine concentrations (Andersen et al., 1999; Huang et al., 1999).

Adenosine is a ubiquitous nucleoside that can be transported across plasma membranes by two classes of nucleoside transporters: *concentrative nucleoside transporter* (CNT) pumps, and *equilibrative nucleoside transporter* (ENT) channels (Nagy et al., 1990; Dunwiddie and Masino, 2001). Additionally, casein kinase 2 (CK2) has been shown to play a role in subtype-specific modulation of ENT action, suggesting it as a regulatory second messenger for ENTs (Stolk et al., 2005). CK2 is an endogenous serine/threonine kinase that preferentially targets specific consensus sequences, one of which is present on the intracellular portion of ENT1 (Bone et al., 2007). Additionally, CK2 has been shown to preferentially phosphorylate ENT1 in neurons (Stolk et al., 2005), and CK2 has high basal levels of activity, but its activity can be modulated (Litchfield, 2003; Ye et al., 2011).

Studies in Chapter 2 showed that a 20min hypoxic insult followed by normoxic reperfusion induced *adenosine-induced post-hypoxia potentiation* (APHP), which is similar to ischemia-induced LTP (i-LTP) found using OGD studies on hippocampal slices. After reperfusion, APHP was prevented by adenosine A2AR inhibitor treatment, indicating that APHP is induced by increased A2AR activity following reperfusion. Adenosine A1R

signaling has been shown to increase during hypoxia, which mediates A1R-mediated synaptic depression (Van Wylen et al., 1986; Coelho et al., 2006; Chen et al., 2014).

In this study, we tested the hypothesis that CK2 regulates the activity of ENTs, which modulate extracellular adenosine tone in the brain. Hippocampal slices were incubated in ENT or CK2 inhibitor drug treatments followed by fEPSP experiments using a selective A1R antagonist (DPCPX), which reveals increased adenosine tone in hippocampal slices treated with ENT and CK2 inhibitors compared to control. Slices with the same treatments were then subjected to a 20min hypoxic insult and a 45min normoxic washout. After 20min hypoxia, hippocampal slices labeled for neurodegeneration with propidium iodide showed neuroprotection with the use of CK2 inhibitor treatments. Additionally, 2 days following PVD surgery, hippocampal slices in PVD rats showed altered adenosine-mediated signaling in CA1, indicating that a small cortical lesion may modulate functioning throughout the brain.

3.3 Methods

3.3.1 Hippocampal slice preparation

Sprague-Dawley rats at postnatal day 18-30 (pn18-30) (Charles River, Canada) were anaesthetized with halothane and decapitated according to protocols approved by the University Committee of Animal Care and Supply (UCACS) at the University of Saskatchewan. Brains were quickly extracted and placed in ice-cold oxygenated dissection medium containing the following (in mM): NaCl, 25 NaHCO₃, 25 glucose, 75 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 7.0 MgCl₂, and 0.5 CaCl₂. Hippocampal slices (400µm thick) were cut using a vibrating tissue slicer (Leica VT1200S, Germany) and maintained for 1h at room temperature in artificial cerebro-spinal fluid (aCSF) containing the following (in mM): 126

NaCl, 2.5 KCl, 2.0 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 2.0 CaCl₂, and aerated with 95% O₂/5% CO₂.

3.3.2 Drug Treatments

Following the 1h equilibration period, hippocampal slices were then treated with one of the following: dimethylsulfoxide (DMSO, vehicle control), dipyridamole (DPY) an ENT1/2 inhibitor (10μM), S-(4-Nitrobenzyl)-6-thioinosine (NBTI), an ENT 1 inhibitor (100nM), or dichlororibofuranosylbenzimidazole (DRB), a CK2 inhibitor (100μM) for 1h. During the experiment, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100nM, A1R antagonist) and SCH442416 (SCH, 5nM, A2AR antagonist) were used as well. All chemicals were obtained from Sigma-Aldrich and diluted as stock solutions in DMSO before being added to aCSF. The final concentration of DMSO was <0.1% in all solutions.

3.3.3 Pial vessel disruption as a model of small-vessel stroke

Disruption of class II size vessels on the surface of the cortex (pia), known as pial vessel disruption (PVD), induces a small cortical lesion that forms a lacuna-like fluid-filled cyst in the area of the lesion within three weeks of PVD surgery (Wang and Walz, 2003; Wang et al., 2004; Cayabyab et al., 2013; Chen et al., 2014). Importantly, this lesion is confined to the cortex, and thus the lesion does not extend into the underlying corpus callosum (Wang and Walz, 2003). Surgeries were performed as described previously (Hua and Walz, 2006). In short, PVD surgery induced an approximately 1mm³ permanent (ie. Non-reperfusion) lesion in the cortex. Rats weighing approximately 250g were anaesthetized with 2% isoflurane and buprenorphine was given for analgesic effects. Anaesthetized rats received a 5mm-diameter craniotomy on the right, rostral side of the bregma adjacent to the coronal sagittal structures. The dura was then opened, and ClassII pial vessels were disrupted using small forceps. The removed piece of skull was then replaced and the wound was closed with a suture clip. Rats

were then given 48h recovery time and were then sacrificed, and hippocampal slices were taken as described above.

3.3.4 Electrophysiology

Following the 1h drug incubation, hippocampal slices were placed in a recording chamber and perfused with aerated aCSF at a rate of 3ml/min. Perfused aCSF contained the same concentration of drug treatment as was used in the pre-incubation treatments. Field excitatory postsynaptic potentials (fEPSPs) were evoked by a tungsten bipolar stimulating electrode, which was placed in CA1 along the Schaffer collateral pathway. fEPSP response was recorded by a glass microelectrode placed in the CA1 stratum radiatum. fEPSP signals were recorded using a Digidata 1440A digitizer (Axon Instruments, Foster City, CA) and saved using Clampex 9.0 (Axon Instruments). fEPSPs were evoked at 30s intervals using a paired pulse stimulation protocol or a single pulse protocol.

Each experiment recorded a stable baseline as in Chapter 2 prior to experimental treatments. A paired-pulse experiment was used to assess adenosine tone and neurotransmitter release with pre-treatment in CK2 and ENT inhibitors. Two pulses 50ms apart were evoked every 30s through the course of the experiment. Following a 20min baseline recording, 100nM DPCPX, a selective A1R antagonist, was perfused in solution for 30min in the presence of the inhibitors listed above. The paired-pulse ratio (PPR) was assessed by finding the ratio of the slope of the second pulse (P2) to the slope of the first pulse (P1) from each measurement. In a subsequent experiment, following a 20min baseline recording, slices were subjected to a 20min hypoxic insult followed by a 45min normoxic washout period. Hypoxia was achieved by bubbling aCSF with 95%N₂/5%CO₂ for a minimum of 20min prior to the experiment starting, and continually through the experiment to deplete oxygen in solution. The gas was balanced with 5% CO₂ to keep pH within a physiological range.

3.3.5 Propidium Iodide Staining

The methods used to stain hippocampal slices with propidium iodide (PI) were adapted from Pugliese et al. (2009). Following equilibration of hippocampal slices for 1h after slicing, the following drug treatments were added: the CK2 inhibitors DMAT and TBB, and the CK2 activator Spermine. Slices that were treated with spermine were also treated with D-APV to prevent aberrant NMDAR activation due to spermine for 1h. Hippocampal slices were then subjected to a 20min hypoxic insult by replacing oxygenated aCSF with hypoxic aCSF that was bubbled with 95%N₂/5%CO₂ prior to, and continuously throughout the hypoxic insult. After 20min hypoxia, aCSF was replaced with normoxic aCSF and the slices were incubated at room temperature for 3h. During the final 1h incubation period, 5µg/ml propidium iodide (Sigma) was added to the aCSF. Following the incubation period, slices were rinsed thoroughly in aCSF and then fixed in 4% paraformaldehyde at 4°C overnight. The following day, slices were washed 3 x 15min in 1X PBS and then mounted on glass microscope slides (VWR) and sealed using Prolong Gold Antifade Reagent (Invitrogen). After the addition of PI, all subsequent procedures were done in the dark to prevent photobleaching.

Hippocampal slices were imaged using a Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss, Germany) using green light (543nm) to induce PI fluorescence. The whole hippocampus was imaged in pieces using a 10x objective lens, and images of CA1 pyramidal neurons were obtained using the Zeiss Plan-Apochromat 63x/1.6 oil objective lens (Carl Zeiss). CA1 images were acquired as Z-stack images of 200µm depth into the hippocampal slice to the outer top of the slice, with each Z-stack image taken at 2µm (total 100µm). Two Z-stack images were taken along CA1 for each slice.

Data was collected using Zeiss Zen 2009 v. 5.5 software (Carl Zeiss) and was analyzed using ImageJ (NIH, public domain). Z-stack images closest to the outer top and bottom of the hippocampal slices were not analyzed, as the neuronal damage in those areas was enhanced

by the slicing procedure. The inner-most 20 μ m segments (~100 μ m down) were combined as maximum intensity projections and intensities were compared between treatment groups. Densitometry analysis was performed on CA1 Z-stack maximum intensity projection images, and densitometry values were normalized to time control slices that were treated along with each experiment. Data was graphed as a percentage of the time control value and analyzed for significance against this control value (100%). Full hippocampal images were assembled as montages of the entire hippocampal slice using Adobe Photoshop CS6 (Adobe Systems, Mountain View, CA).

3.4 Results

3.4.1 CK2 and ENT inhibition increased adenosine tone in CA1 of rat hippocampal slices

To assess the functional effect of ENT and CK2 inhibition on adenosine tone and the probability of neurotransmitter release, we performed paired-pulse fEPSP recordings in CA1 of rat hippocampal slices that were pre-incubated in NBTI (100 μ M), DRB (100 μ M), or DPY (10 μ M) followed by inhibition of A1Rs using the selective antagonist DPCPX (100nM). We hypothesized that inhibition of CK2 and ENT1/ENT2 would inhibit adenosine transport and thus increase extracellular adenosine tone. Blockade of A1Rs was therefore hypothesized to increase synaptic transmission in hippocampal slices pre-treated in CK2 and ENT inhibitors. As shown in Figure 3.1, during perfusion of DPCPX, fEPSPs were significantly increased with both ENT and CK2 inhibitor treatments compared to control (DMSO). Blockade of A1Rs removes A1R-mediated synaptic depression both presynaptically and postsynaptically. Results indicated an increase in both adenosine concentration and A1R activation. The paired-pulse ratio (PPR) decreases significantly more than control with NBTI, DPY, and DRB incubated hippocampal slices (Figure 3.1C), indicating paired-pulse depression (PPD), or an increase in the probability of neurotransmitter release, when treated with 30min

DPCPX. These results indicate that both CK2 and ENT inhibition increases adenosine tone and that both proteins are involved in the regulation of basal adenosine tone in the hippocampus.

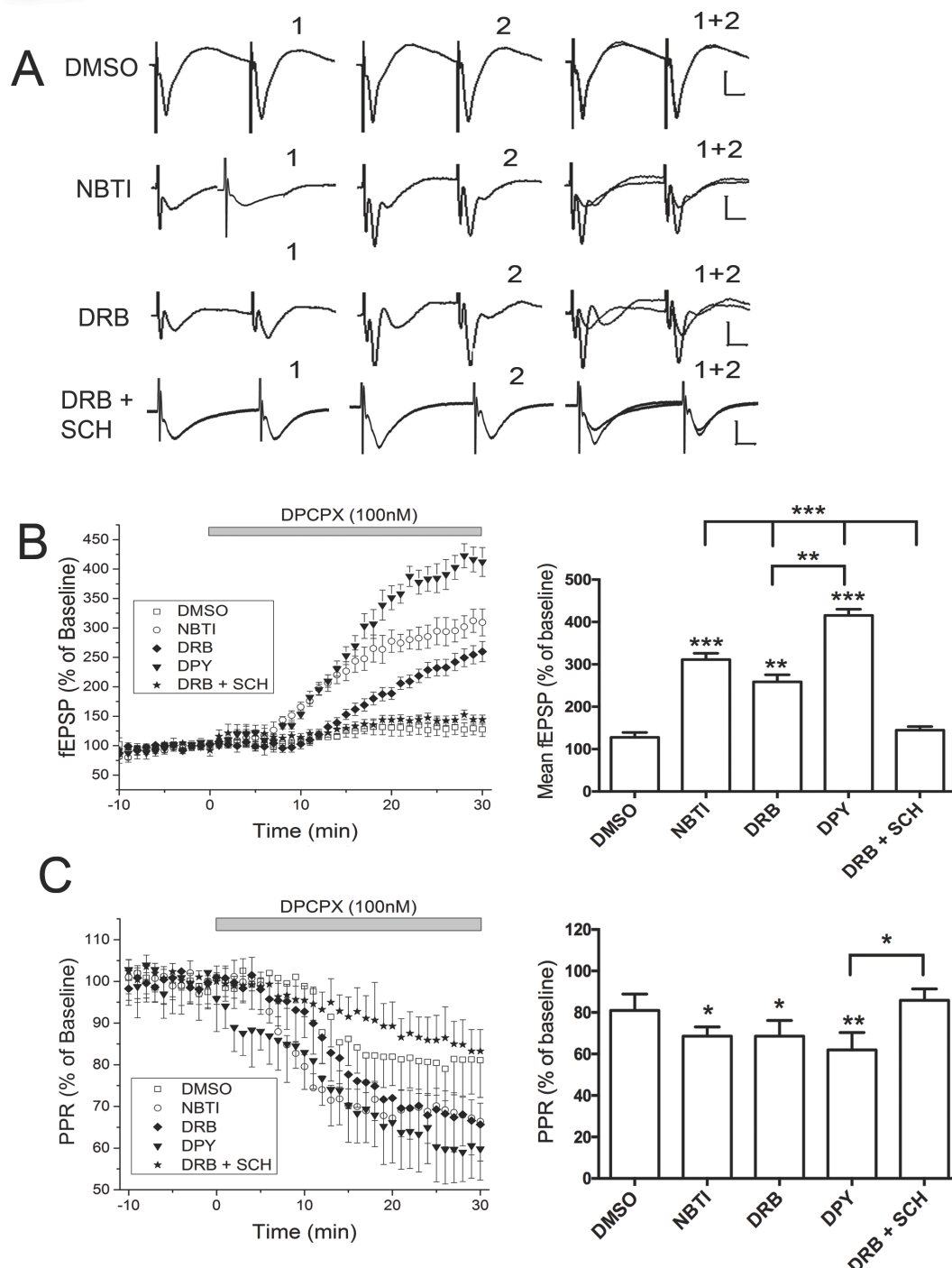


Figure 3.1. CK2 and ENT inhibition increased adenosine tone in hippocampal slices.

Paired-pulse fEPSP recordings in CA1 of rat hippocampal slices pre-treated with CK2

inhibitor (DRB, 100 μ M), ENT1 inhibitor (NBTI, 100nM), or ENT1/ENT2 inhibitor (DPY, 10 μ M) and perfused with DPCPX (100nM) for 30min. (A) Representative fEPSP traces for treatment groups DMSO (control), NBTI, and DRB. 1 (Left): Average paired-pulse traces as an average of the final 10 sweeps during baseline recording. 2 (Middle): Average paired-pulse traces of the final 10 sweeps of 30min DPCPX treatment. 1+2 (Right): Overlay of baseline and DPCPX treatments together. Scale bars represent 0.25mV, 10ms. (B) Left. Time course plot of fEPSP slope (10min baseline, 30min DPCPX treatment) normalized to the final 10 sweeps of baseline recording (100%). After 30min DPCPX, fEPSPs were significantly potentiated between 300-500% of baseline with CK2 and ENT inhibitor pre-treatment and no significant change in DMSO. Right. Summary bar chart shows CK2 and ENT inhibitors significantly increased fEPSP slope after 30min DPCPX treatment. (C) Left. Time course plot of paired-pulse ratio (PPR) over course of the experiment. During baseline (1) PPR was approximately 1 (100%) for each treatment group. After 30min DPCPX treatment (2) PPR was significantly reduced (paired-pulse depression, PPD) in CK2/ENT inhibitor pre-treated groups. Right. Summary bar chart showing that CK2 and ENT inhibitor pre-treatment significantly reduced PPR after 30min DPCPX treatment, indicating PPD. Average values are mean \pm SEM, n=3-6 independent experiments, each from different rats, * P<0.05, ** P<0.01 and *** P<0.001..

3.4.2 ENT1/2 inhibition reduced hypoxia-induced synaptic depression in hypoxia and ENT1/2 and CK2 inhibition reduced post-hypoxic potentiation in CA1 of rat hippocampal slices

To examine the functional effects of enhanced reduction of surface expression of ENTs and A1Rs with 20min hypoxia in the presence of ENT and CK2 inhibitors found within this lab, we performed fEPSP recordings on hippocampal slices pre-treated in NBTI, DRB, or

DPY with a 20min hypoxic insult followed by a 45min normoxic washout period. Figure 3.2 shows that both treatment with DPY, an ENT1 and ENT2 inhibitor, and DRB, a CK2 inhibitor, significantly reduced hypoxia-induced synaptic depression compared to control (DMSO) and NBTI (ENT1 inhibitor) treatments. NBTI did not significantly affect hypoxia induced synaptic depression.

During normoxic washout (Figure 3.2B,C), control CA1 fEPSPs rapidly recovered to baseline and continued to increase and become potentiated significantly above baseline levels. This post-hypoxic potentiation was reduced significantly with DPY, DRB, and NBTI treatment. This suggests that ENT1/ENT2 and CK2 activity during hypoxia reperfusion is involved in potentiation of neurotransmission in the hippocampus. These results indicate a correlation between decreased A1R and ENT surface expression with CK2 and ENT inhibition and reduced hypoxia-induced synaptic depression.

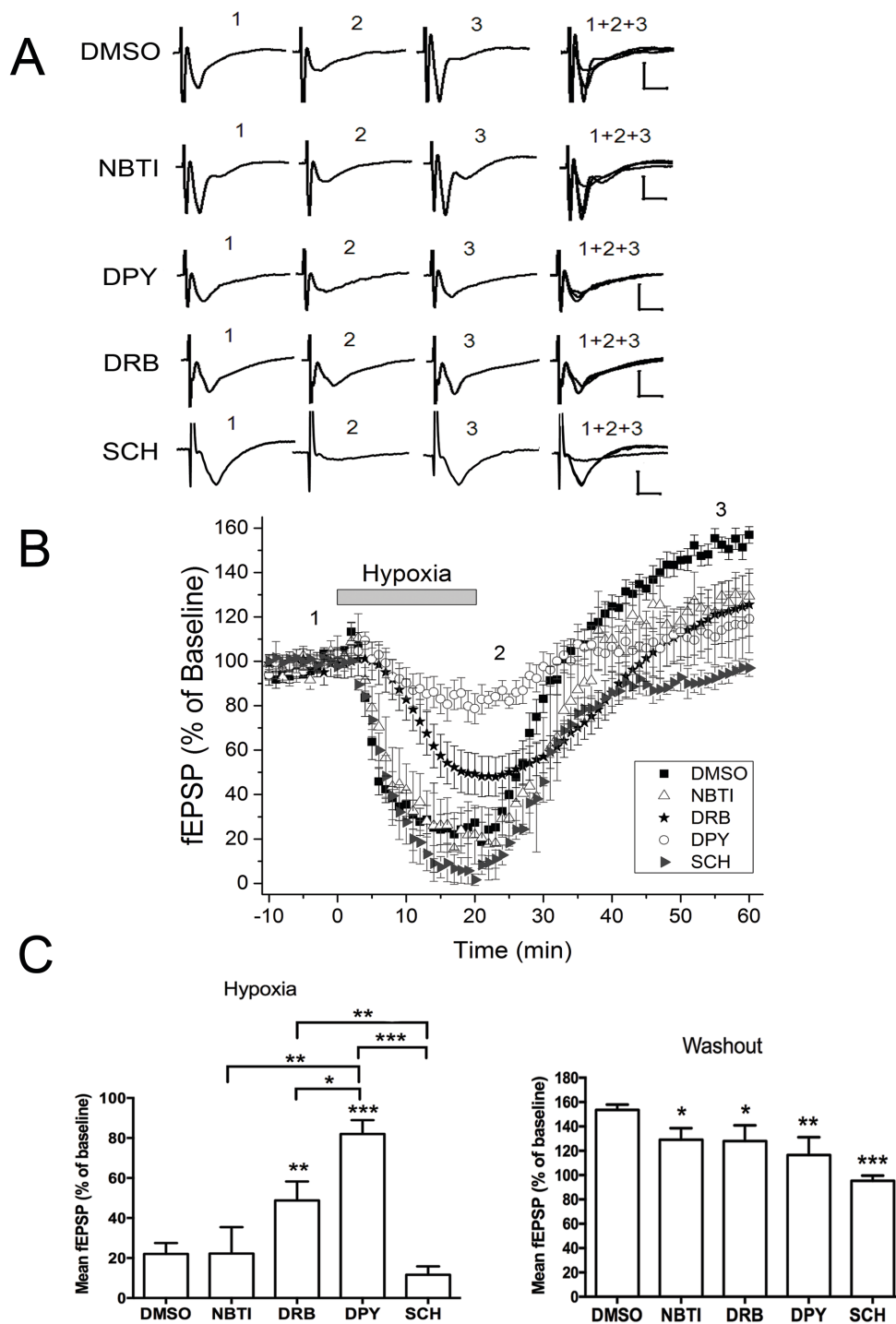


Figure 3.2. CK2 and ENT1/ENT2 inhibitors reduced hypoxia-induced synaptic depression and CK2 and ENT inhibitors decreased potentiation during normoxic reperfusion. (A) Representative fEPSP traces for each treatment group: DMSO (control), NBTI (ENT1 inhibitor, 100nM), DPY (ENT1/ENT2 inhibitor, 10 μ M), DRB (CK2 inhibitor, 100 μ M), and SCH (A2AR inhibitor, 5nM). 1. Baseline fEPSP traces. 2. fEPSP traces with

20min hypoxia treatment. 3. fEPSP traces after 45min normoxic washout. 1+2+3. Overlay of representative traces for each treatment group shown to the left. Scale bars represent 0.25mV, 10ms. (B) Time course plot showing average data for each treatment group throughout the course of the experiment. 1. Final 10min of baseline recording. 2. Hypoxia treatment period (20min). 3. Normoxic washout period. During hypoxia, both DPY and DRB significantly reduced hypoxia-induced synaptic depression compared to control (DMSO) and NBTI. During normoxic washout, inhibitor treatments (NBTI, DPY, DRB) reduced post-hypoxic potentiation seen in control. (C) Summary bar charts showing the mean of the final 10 traces from 20min hypoxia (left), and 45min normoxic washout (right) normalized as a percentage of baseline levels (100%). In hypoxia, DRB and DPY both significantly reduced the amount of synaptic depression compared to both DMSO and NBTI. In washout, NBTI, DRB, SCH, and DPY significantly reduced potentiation seen in control. Average values are mean \pm SEM, n=6-12 independent experiments from different rats, * P<0.05, ** P<0.01 and *** P<0.001.

3.4.3 CK2 inhibition provided neuroprotection to hippocampal slices after 20min hypoxia.

The role of CK2 as a potential neuroprotective protein has been explored (Kim et al., 2009), but the direct effect of CK2 inhibition or activation in neuroprotection or neurodegeneration in hypoxia or ischemia have not been well established. To test the effect of CK2 in hypoxia-induced neurodegeneration, hippocampal slices were pre-treated with DMAT or TBB, which are both CK2 inhibitors, or with Spermine, which is a CK2 activator, or, due to the ability for spermine to also activate NMDA receptors by direct interaction, we also treated slices with Spermine + D-APV, an NMDAR inhibitor. Following a 20min hypoxic insult, slices were reintroduced to normoxic conditions and incubated for 3h prior to fixation. In the final hour of incubation, propidium iodide (PI) was added to label dead cells for neurodegeneration. Figure 3.3 shows that hypoxia alone induced significant

neurodegeneration (Figure 3.3B), whereas slices treated with DMAT and TBB showed significantly less neurodegeneration than hypoxia alone (Figure 3.3C-E). Time control slices showed significantly less neurodegeneration than hypoxia control (DMSO), and densitometry analysis was performed with densitometry values normalized to time control (100%). DMAT and TBB treatment prior to hypoxia were shown to be neuroprotective, while spermine did not prevent hypoxia-induced neurodegeneration. Spermine with the NMDAR inhibitor D-APV was also neuroprotective, indicating that CK2 activation may contribute to neurodegeneration through increasing NMDAR activity. These data suggest that inhibition of CK2 activity, or reduced CK2 activation, affords neuroprotection to hippocampal neurons.

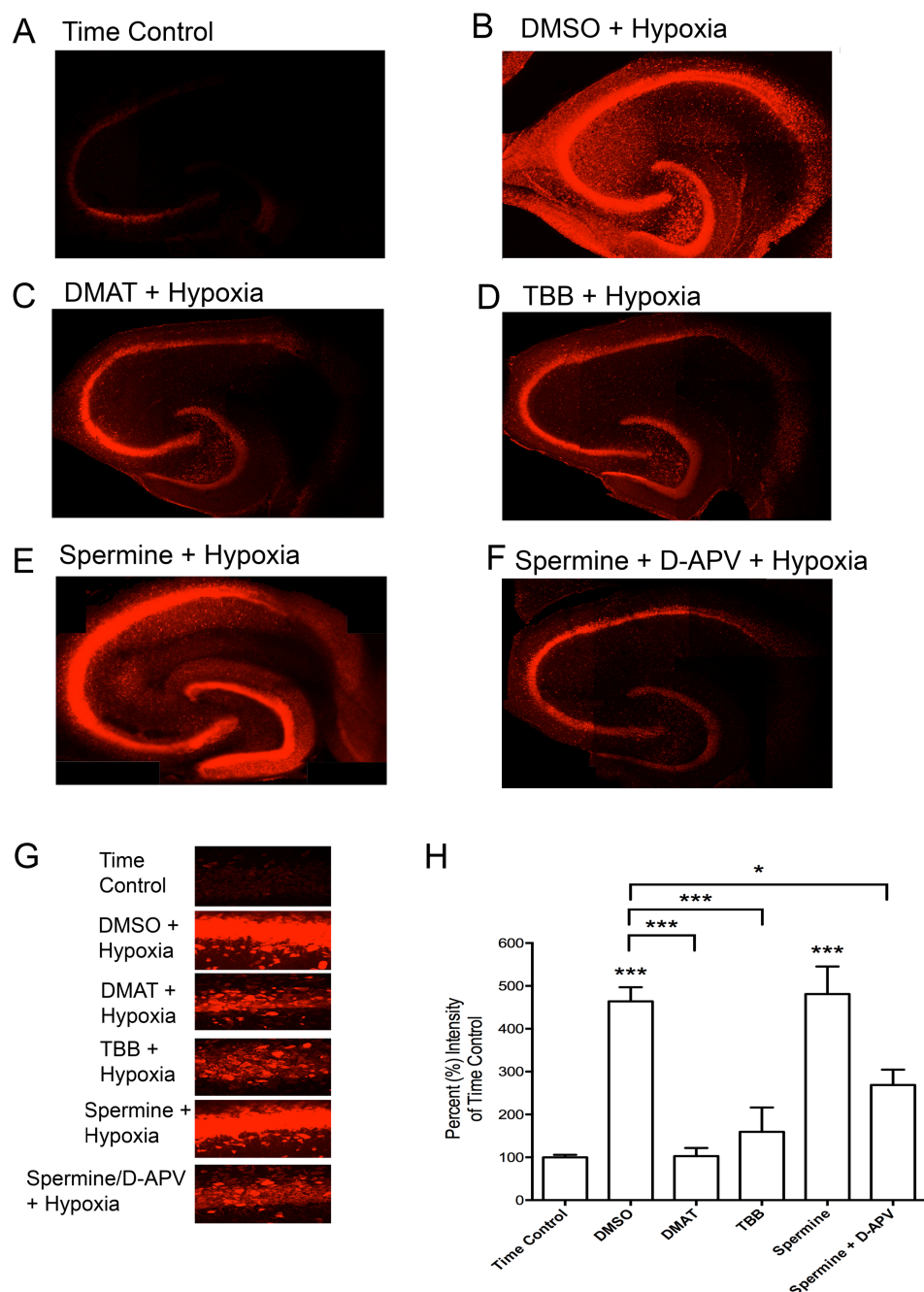


Figure 3.3. CK2 inhibitor treatment reduced neurodegeneration caused by a 20min hypoxic insult in hippocampal slices. (A) Representative propidium iodide staining images of time control hippocampal slices, which were not subjected to any drug treatments or hypoxic conditions. There is very little neurodegeneration seen in these slices, and all treated slices were normalized to the time control values (100%). (B) Slices treated with DMSO with hypoxia showed significant neurodegeneration approximately 450% of time control values.

(C) Slices treated with DMAT showed significantly less neurodegeneration compared to DMSO. (D) Representative slice treated with the CK2 inhibitor TBB. (E) Representative hippocampal images showing increased neurodegeneration in slices treated with spermine alone. (F) Representative slice showing treatment with spermine and D-APV. (G) Summary bar graph showing values of average intensity normalized to time control values (100%). Average values are shown as mean \pm SEM with arbitrary units, n=4 independent experiments, 5 rats per experiment. Significance values * $P < 0.05$ and *** $P < 0.001$.

3.4.4 Focal cortical ischemia in an in vivo PVD small-vessel stroke model contributes to tonic synaptic depression by increasing adenosine tone in the hippocampus.

Many focal cerebral ischemia models involve occlusion of large cerebral blood vessels such as the middle cerebral artery, which results in damage to the striatum and cortex to varying degrees depending on the duration of vessel occlusion (Traystman, 2003). During hypoxia, transient global ischemia or focal cerebral ischemia, it is well accepted that there is an increase in the extracellular levels of adenosine. Brain damage in global and focal ischemia models occurs within selectively vulnerable areas such as the hippocampal CA1 region, neocortex, and striatum. Global ischemia has been shown to selectively reduce the expression of GluA2-containing AMPARs in the CA1 region in rats (Sommer and Kiessling, 2002). In this study, we have used a modified pial vessel disruption (PVD) protocol, which mimics mild, small-vessel strokes. This involves permanent disruption of class II size pial vessels, and has been shown to produce a consistent cone-shaped cortical lesion damage that does not extend to the corpus callosum (Wang and Walz, 2003; Hua and Walz, 2006).

In this study, we hypothesized that adenosine surges in the brain will be sufficiently prolonged to induce increased adenosine tone in the hippocampus 48h post-PVD surgery.

Since it is widely accepted that adenosine is tonically elevated during cerebral ischemia (Dale et al., 2000), we tested the hypothesis that adenosine surges in the brain after permanent focal disruption of small cortical pial vessels may be sufficient to affect vulnerable brain regions, such as the hippocampus, and influence the induction of synaptic depression. Therefore, we evaluated the effects of PVD vs. sham surgeries on synaptic transmission two days post surgeries. The fEPSP recordings from hippocampal slices were obtained from the ipsilateral side of PVD surgery lesion or sham surgery. Consistent with a downregulation of A1Rs after PVD, we observed less synaptic potentiation and paired-pulse depression when the A1R antagonist DPCPX was applied to the PVD slices compared to sham brains (Figure 3.3A-B).

independent experiments (4 animals each), with * $P < 0.05$ and ** $P < 0.01$ unpaired Student's *t*-test. Calibration: 0.5mV, 5ms in A, and 10ms in B. The numbers "1" and "2" associated with figure traces and time course charts correspond to fEPSPs at baseline and fEPSP in DPCPX, respectively.

3.5 Discussion

This chapter focused on investigating a functional ENT/CK2 interaction, potential neuroprotection through CK2 inhibition, and alterations in adenosine tone 48h following PVD surgery. Both ENT1 and ENT2 are expressed ubiquitously in mammalian cells, and are distributed throughout the central nervous system, including the hippocampus (Jennings, 2001). Little is known about the regulation of these transporters, but they have been shown to play a role in the regulation of adenosine levels in physiological and ischemic conditions (Zhang et al., 2011). ENT1 and ENT2 have a large intracellular loop joining transmembrane domains 6 and 7, where putative CK2 target consensus sequences are located (Stolk et al., 2005). These phosphorylation sites suggest that ENT transporter activity and/or membrane expression may be subject to modulation by CK2.

The present study shows that CK2 inhibition with DRB, ENT1 inhibition with NBTI, or ENT1/2 inhibition with DPY significantly increased adenosine tone, which corresponds to significantly reduced ENT surface expression seen after CK2 inhibition. These results indicate that CK2-mediated phosphorylation of ENTs contributes to the regulation of extracellular adenosine levels in CA1 of the hippocampus. This represents a potentially important mechanism for neuroprotective therapy in hypoxic/ischemic neuronal damage. To test this potential, propidium iodide-stained slices showed that CK2 inhibitors were neuroprotective following a 20min hypoxic insult (Figure 3.3). CK2 activity has been shown to decrease in the hippocampus during ischemic injury, and preventing this decrease has

been shown to be neuroprotective (Lee et al., 2004). In contrast, CK2 activity increases in brain regions that are resistant to ischemic damage (Bone et al., 2007). This may indicate that an endogenous signal, may reduce CK2 levels in ischemic/hypoxic conditions as a protective mechanism to enhance neuronal survival.

ENTs are the most abundant adenosine transporters and control adenosine levels in the brain (Noji et al., 2004), and ENT inhibition has been shown to increase extracellular adenosine in the hippocampus (Dunwiddie and Masino, 2001). In addition, inhibition of adenosine uptake by NBTI and DPY (ENT1 and ENT1/2 inhibitors, respectively) cause increased adenosine levels in the olfactory bulb (Sanderson and Scholfield, 1986). Here, we show that hippocampal slices pre-incubated with CK2/ENT inhibitors, potentiated fEPSPs after 30min DPCPX treatment, indicated that there was higher extracellular adenosine and more A1R activation in these slices compared to the control (Figure 3.1). This suggests that reduced ENT and CK2 function correspond to increased extracellular adenosine tone in basal conditions. With the paired pulse ratios, there was paired-pulse depression in the inhibitor-treated slices, showing that after DPCPX treatment, there was an increase in the probability of neurotransmitter release, showing a presynaptic involvement in both ENTs and CK2 in altering adenosine A1R function presynaptically. Because the A1R has a higher binding affinity (K_d) for adenosine (70nM) than A2AR (150 nM) (Sanderson and Scholfield, 1986), increased adenosine levels by ENT inhibition may predominantly affect A1R-mediated synaptic depression but since it also showed increased adenosine tone, the A2A receptor could be involved in these excitatory actions. Further studies with the use of A2A receptor inhibition would be required to fully elucidate this mechanism.

The reduced synaptic depression during hypoxia in hippocampal slices pre-treated with CK2 and ENT inhibitors indicates that reduced A1R and ENT surface expression after 20min hypoxia corresponds to reduced A1R-mediated synaptic depression in hypoxic conditions. So

inhibition of CK2 may increase adenosine tone via reduction of ENT1 phosphorylation, which induce reduction in ENT1 surface expression and activity (Handa et al., 2001). We tested both CK2 inhibitors (DMAT and TBB) and CK2 activator (Spermine or Spermine + D-APV) to test potential neuroprotection in hypoxia in hippocampal slices that were stained with propidium iodide. DMAT, TBB and Spermine all showed neuroprotection compared to hypoxia alone (Figure 3.3).

Finally, using hippocampal slices taken from rats 48h post-PVD surgery, fEPSP experiments showed reduced synaptic potentiation following A1R inhibition with DPCPX. This reduced potentiation could indicate that there is less inhibitory tone, possibly due to increased A1R desensitization (Coelho et al., 2006) or increased A2AR activity. Importantly, these results show that small focal cortical ischemia induces increased adenosine levels in the hippocampus, indicating a global effect of focal ischemia in the brain. Further studies are required to further elucidate the role of increased adenosine tone in the post-ischemic brain after focal ischemia.

In conclusion, this chapter suggests a role for CK2 in the regulation of adenosine levels in the hippocampus. CK2 and ENTs were also shown to be involved in the CA1 hippocampal response to hypoxia/reperfusion injury, and CK2 modulation with drug treatments provided hippocampal slices with neuroprotection. These results indicate that CK2 may be a viable neuroprotective target in ischemia. The implications of a global changes in adenosine signaling after focal cortical ischemia may mediate neurodegeneration and neurological deficits which can occur after stroke, such as post-stroke dementia (Zhao et al., 2001).

Chapter 4: Summary and Conclusions

4.1 Summary and Discussion

This thesis explores novel mechanisms contributing to adenosine-mediated neuromodulation in the hippocampus in hypoxia and in PVD-induced ischemia. These mechanisms suggest potential targets for the development of neuroprotective strategies to mitigate ischemia-induced brain damage. Using both fEPSP electrophysiology and biotinylation Western blotting techniques using rat hippocampal slices that were given various pharmacological treatments, we identify a novel role for PP1, PP2A and PP2B in adenosine and hypoxia-induced synaptic modulation in the hippocampus. Propidium iodide staining on slices subjected to a hypoxic insult also showed that these protein phosphatases mediated hypoxia-induced neurodegeneration while protein phosphatase 1, 2A and 2B inhibition was shown to be neuroprotective following a 20min hypoxic insult.. PP1, PP2A, and PP2B were shown to regulate A1R-induced GluA1 internalization and subsequent A1R-induced synaptic depression, whereas only PP2A prevented A1R-induced GluA2 internalization. After a 20 minute hypoxic insult, normoxic reperfusion induces significant *adenosine-induced post-hypoxic potentiation* (APHP), which is dependent on A2AR-mediated increased GluA1 expression through PKA activation (Dias et al., 2012). Functional fEPSP data shows that inhibition of CK2 has similar effects as inhibition of ENT1 and ENT2 by increasing adenosine tone and modulating synaptic activity responses during a 20 minute hypoxic insult. The data presented in Chapter 3 support the hypothesis that CK2 phosphorylation of ENT1 regulates the function of ENTs which in turn regulates extracellular adenosine tone in the hippocampus. Inhibition of CK2 also provides neuroprotection to hippocampal neurons in hypoxia. Finally, PVD focal cortical ischemia induced changes in adenosine signaling in CA1 of hippocampal slices 2 days after PVD induction.

These studies emphasize the importance of adenosine-mediated neuromodulation in hypoxic or ischemic conditions, and identify PP1, PP2A, and PP2B as intracellular mediators

of adenosine A1 and A2A receptor-induced neuromodulation in the hippocampus during hypoxic brain injury. Figure 4.1 summarizes some major mechanisms examined in this thesis and the regulatory effects of adenosine in hippocampal neurons.

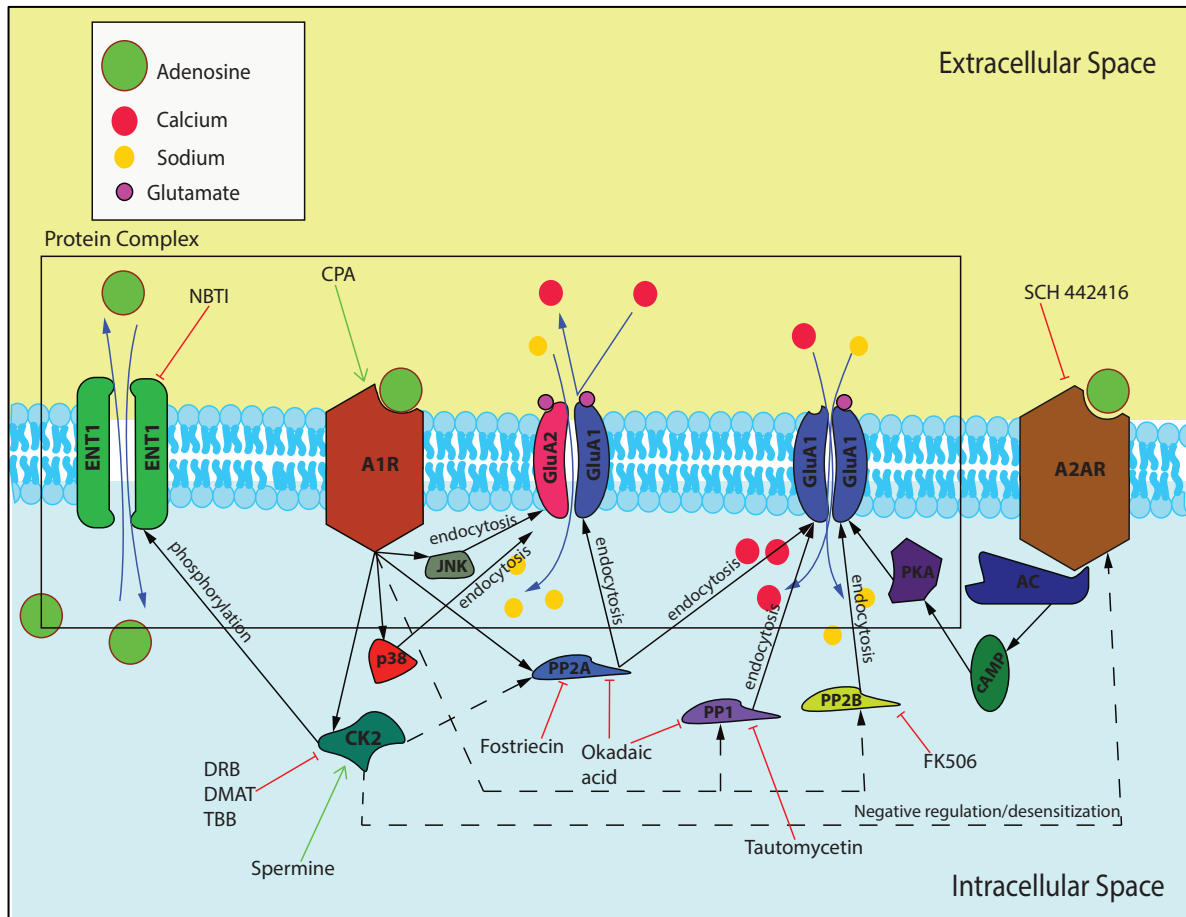


Figure 4.1. Summary of postsynaptic membrane signaling mechanisms examined in this thesis. Important summary of the key receptors/proteins and their important physiological functions that were elucidated in this thesis and previous publications (Rebholz et al., 2009; Chen et al., 2014). Solid black lines indicate the direction of a protein's action on another, and unless otherwise shown, black arrows indicate activation. Dashed lines indicate that either a direct or indirect interaction is occurring, and more research is required to describe whether there are intermediate steps in these signals. Dark blue arrows indicate the

movement of a molecule through a channel. Red lines indicate drug inhibitors and green lines indicate drug agonists.

Following ischemic or hypoxic insults to the hippocampus, the particularly vulnerable CA1 region is prone to profound synaptic depression and neurodegeneration (Pellegrini-Giampietro et al., 1992; Dale et al., 2000). Due to the essential role of the hippocampus in multiple learning and memory processes, neurodegeneration in this area can have a widespread deleterious effects on memory and cognition, and has been linked to a condition known as post-stroke dementia (Zhao et al., 2001). Development of neuroprotective treatments is imperative and of the utmost concern (Gladstone et al., 2002). Recent developments show that adenosine is a major neuromodulator during noxious events in the brain, and defining the widespread actions of adenosine through the family of adenosine receptors may lead to novel targets for neuroprotection in stroke and other neurodegenerative disorders (Boison, 2006; Shen et al., 2011; Pedata et al., 2013). Based on the hypothesis that small strokes may contribute to increased risk of post-stroke dementia (Zhao et al., 2001)

Adenosine-mediated changes in the hippocampus through the intracellular actions of the adenosine A1 (A1R) and A2A (A2AR) receptors are the main focus of this thesis. Through utilizing multiple pharmacological agents along with hypoxia experiments on hippocampal slices and PVD focal cortical ischemia, the effects of three major serine/threonine phosphatases, PP1, PP2A and PP2B were shown to mediate adenosine A1R-induced synaptic depression through producing AMPA receptor subunit GluA1, and to a smaller extent GluA2, internalization in the hippocampus. Additionally, CK2 inhibition was shown to have similar effects as equilibrative nucleoside transporter (ENT) inhibition, which was to increase extracellular adenosine tone, thus increasing A1R-mediated synaptic depression in CA1 of rat

hippocampal slices. Finally, CK2 inhibition was shown to be neuroprotective after 20min hypoxia.

In chapter 2, we investigated the role of protein phosphatases 1, 2A and 2B in adenosine-mediated synaptic depression with selective A1R agonist treatment and hypoxia/reperfusion injury in rat hippocampal slices. These three phosphatases were shown to mediate A1R-induced GluA1 endocytosis by modulating the phosphorylation of Ser831 and Ser845 GluA1 C-terminal residues, two known regulatory sites of GluA1 which modulate GluA1 surface expression and synaptic translocation (Roche et al., 1996; Lee et al., 2003). Protein phosphatase 1, 2A and 2B inhibition reduced CPA-induced GluA1 internalization, but only PP2A inhibition prevented CPA-induced GluA2 endocytosis. Along the same trend, the levels of GluA1 pSer831 and pSer845 were reduced by CPA, and this reduction in pSer831 and pSer845 was prevented by pre-treatment of hippocampal slices with PP1, PP2A and PP2B inhibitors. Along with reducing AMPAR surface expression, 30min A1R activation induced AP5D (Chen et al., 2014), which was reduced by protein phosphatase inhibitor pre-treatment. Taken together with previous work, p38 MAPK and JNK along with PP1, PP2A and PP2B appear to have differential regulatory actions on GluA1- and GluA2-containing AMPAR surface expression (Chen et al., 2014). Importantly, we show that protein phosphatase inhibitor treatment to hippocampal slices promotes neuroprotection in the hippocampus following 20min hypoxia (Figure 2.8), implicating protein phosphatase activity induced by A1R activation in pro-apoptotic intracellular signaling.

Although adenosine A1Rs have been accepted as a neuroprotective adenosine receptor, these data suggest that although acute A1R-induced synaptic depression in ischemia may be neuroprotective, the long-term intracellular changes induced by A1R activation induce neurodegeneration and that inhibition of A1R-activated intracellular proteins provides neuroprotection to hippocampal neurons. We suggest that chronic changes caused by

adenosine A1R signaling may enhance neurodegeneration in the hippocampus. These studies show that inhibition of PP1, PP2A, and PP2B were neuroprotective and prevented A1R-induced GluA1 internalization.

To examine these effects in hypoxia-reperfusion conditions, hippocampal slices were subjected to a 20min hypoxic insult followed by a 45min normoxic washout/reperfusion period. After hypoxia, both control and protein phosphatase inhibitor-treated hippocampal slices showed fEPSP recovery in normoxic reperfusion above baseline, or APHP (Figure 2.4). Treatment with SCH442415 (5nM), an A2AR antagonist, prevented APHP (Figure 2.7), indicating that A2AR activation following reperfusion mediates APHP. Slices subjected to 20min hypoxia followed by 45min normoxic washout showed increased surface GluA1, but not GluA2, levels during reperfusion-induced APHP, which was prevented by pre-treatment with SCH442416 (Figure 2.5). Increased GluA1 surface expression after 45min washout also corresponded to increased pSer831 and pSer845 levels, which was also prevented with SCH442416 treatment (Figure 2.6). This thesis describes a novel mechanism where PP1, PP2A, and PP2B mediate the functional interaction between A1Rs and GluA1, where protein phosphatases induced GluA1-containing AMPARs. It also suggests a pro-neurodegenerative role of excess protein phosphatase activity in neuronal insult conditions. Previous studies have described the involvement of PP1, PP2A and PP2B in various hypoxia, oxygen-glucose deprivation, or ischemia models (Rundén et al., 1998; Cid et al., 2007), and their inhibition has been implicated in neuroprotection. In this study, we add to the wide list of functions performed by these three ubiquitous protein phosphatases.

Recently, Dias et al. showed that 10min oxygen-glucose deprivation (OGD) to hippocampal slices caused reduced excitatory postsynaptic currents (EPSCs) followed by increased recovery above baseline levels (potentiation), which has been termed *ischemia-induced long-term potentiation* (i-LTP) (Dias et al., 2013). The results from fEPSP hypoxia

studies performed in this thesis mimicked the effect seen by Dias et al. (2013), showing significant synaptic depression in hypoxia followed by fEPSP potentiation during normoxic reperfusion. Both i-LTP and APHP involve A2AR activation and increased CP-AMPA activity, and potentiation can be reduced by either A2AR inhibition or CP-AMPA inhibition (Dias et al., 2013). These data indicate that GluA1-containing AMPARs and A2ARs are key mediators of reperfusion injury to neurons by inducing increased Ca^{2+} permeability and cellular excitability, thus increasing the chance of excitotoxicity. Additionally, hypoxia reduced GluA2 surface expression which did not increase after reperfusion. Reduced GluA2 surface expression has been associated with neurodegeneration through apoptotic pathways (Pellegrini-Giampietro et al., 1997)

Although the mechanism by which adenosine induces neuromodulatory effects in the hippocampus are not fully understood, this study further emphasizes the importance of this essential molecule in hypoxic insult conditions in the hippocampus. GluA1 is the most abundantly expressed AMPAR subunit and confers Ca^{2+} -permeability (Rogers et al., 1991). Increase CP-AMPA surface expression is a known mechanism for the induction of LTP (Szabo et al., 2012), which shows similarities to i-LTP, except that CP-AMPA expression is transient in LTP, whereas it is more prolonged in i-LTP (Szabo et al., 2012), which may explain why CP-AMPA expression is not neurotoxic in LTP induction.

The experiments performed in Chapter 3 further investigate adenosine-induced neuromodulation through the regulation of extracellular adenosine by ENTs. Since ENTs are passive and equilibrative, their action is largely regulated by the modulation of their surface expression (Parkinson et al., 2011). Since ENT1 contains CK2 consensus sequences, it follows that CK2 may play a role in the adenosine-induced modulation of ENT1 surface expression, which when reduced as it is in ischemia, may enhance neurodegeneration (Zhang et al., 2011). CK2 also provides a potential intermediary protein for the communication

between adenosine A1Rs and ENT1 in the hippocampus (Bone et al., 2007). Results suggest that CK2 activity in hypoxia contribute to neurodegeneration following hypoxia, whereas inhibition of CK2 prevents hypoxia-induced neurodegeneration. During hypoxia, A1Rs become desensitized (Dixon et al., 1997; Coelho et al., 2006), which would reduce A1R-mediated effects including increased CK2 activity. CK2 is a negative regulator of A2ARs (Rebholz et al., 2009), meaning that reduced CK2 activity increases A2AR activity. These results indicate that CK2 may mediate a sort of cross-talk between A1 and A2A receptors.

4.2 Future directions

One of the most important immediate future directions is to further classify the intracellular mechanisms described in this research by using a specific tat-peptide knockdown technique both in acute hippocampal slices and in PVD stroke. In fact, we are currently exploring these mechanisms using a tat-peptide interference technique with peptides YP and YD, which are designed to inhibit the function of CK2 and PP2A, respectively.

Additionally, studies should be performed to examine the potential neuroprotective effects of the drug inhibitors used in these studies by classifying hypoxia- and ischemia-induced neurodegeneration in the hippocampus. Similar propidium iodide staining experiments should also be done with 30min CPA treatment to test whether CPA induces any neurodegeneration. Finally, the role of CK2 as a potential mediator of A1R and A2AR cross-talk will be further explored using fEPSP, confocal imaging and Western blotting techniques.

References

References

- Abbracchio MP, Brambilla R, Ceruti S, Kim HO, Von Lubitz DK, Jacobson KA, Cattabeni F (1995) G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. *Molecular pharmacology* 48:1038-1045.
- Ai H, Yang W, Ye M, Lu W, Yao L, Luo J-h (2011) Differential regulation of AMPA receptor GluA1 phosphorylation at serine 831 and 845 associated with activation of NMDA receptor subpopulations. *Neuroscience letters* 497:94-98.
- Ali M, Fulton R, Quinn T, Brady M, Lees KR, Alexandrov A, Bath PM, Bluhmki E, Bornstein N, Claesson L (2013) How well do standard stroke outcome measures reflect quality of life? a retrospective analysis of clinical trial data. *Stroke* 44:3161-3165.
- Allen PB, Hvalby O, Jensen V, Errington ML, Ramsay M, Chaudhry FA, Bliss TV, Storm-Mathisen J, Morris RG, Andersen P (2000) Protein phosphatase-1 regulation in the induction of long-term potentiation: heterogeneous molecular mechanisms. *The Journal of neuroscience* 20:3537-3543.
- Alzheimer C, Kargl L, Bruggencate GT (1991a) Adenosinergic inhibition in hippocampus is mediated by adenosine A1 receptors very similar to those of peripheral tissues. *European journal of pharmacology* 196:313-317.
- Alzheimer C, Rohrenbeck J, Ten Bruggencate G (1991b) Adenosine depresses induction of LTP at the mossy fiber-CA3 synapse in vitro. *Brain research* 543:163-165.
- Andersen BT, Gillespie DG, Mi Z, Dubey RK, Jackson EK (1999) Role of adenosine A1 receptors in modulating extracellular adenosine levels. *Journal of Pharmacology and Experimental Therapeutics* 291:76-80.
- Anderson CM, Xiong W, Geiger JD, Young JD, Cass CE, Baldwin SA, Parkinson FE (1999) Distribution of equilibrative, nitrobenzylthioinosine-sensitive nucleoside transporters (ENT1) in brain. *Journal of neurochemistry* 73:867-873.
- Ankarcrona M, Dypbukt JM, Orrenius S, Nicotera P (1996) Calcineurin and mitochondrial function in glutamate-induced neuronal cell death. *FEBS letters* 394:321-324.
- Asai A, Qiu J-h, Narita Y, Chi S, Saito N, Shinoura N, Hamada H, Kuchino Y, Kirino T (1999) High level calcineurin activity predisposes neuronal cells to apoptosis. *Journal of Biological Chemistry* 274:34450-34458.
- Baldwin SA, Beal PR, Yao SY, King AE, Cass CE, Young JD (2004) The equilibrative nucleoside transporter family, SLC29. *Pflügers Arch* 447:735-743.
- Barria A, Derkach V, Soderling T (1997) Identification of the Ca²⁺/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor. *J Biol Chem* 272:32727-32730.
- Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, von Zastrow M, Malenka RC (2000) Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nature neuroscience* 3:1291-1300.
- Belmeguenai A, Hansel C (2005) A role for protein phosphatases 1, 2A, and 2B in cerebellar long-term potentiation. *The Journal of Neuroscience* 25:10768-10772.
- Benarroch EE (2008) Adenosine and its receptors: multiple modulatory functions and potential therapeutic targets for neurologic disease. *Neurology* 70:231-236.
- Bialojan C, Takai A (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem J* 256:283-290.
- Bochelen D, Rudin M, Sauter A (1999) Calcineurin inhibitors FK506 and SDZ ASM 981 alleviate the outcome of focal cerebral ischemic/reperfusion injury. *Journal of Pharmacology and Experimental Therapeutics* 288:653-659.

- Boison D (2006) Adenosine kinase, epilepsy and stroke: mechanisms and therapies. *Trends in pharmacological sciences* 27:652-658.
- Bone DB, Robillard KR, Stolk M, Hammond JR (2007) Differential regulation of mouse equilibrative nucleoside transporter 1 (mENT1) splice variants by protein kinase CK2. *Mol Membr Biol* 24:294-303.
- Brundege JM, Dunwiddie TV (1996) Modulation of excitatory synaptic transmission by adenosine released from single hippocampal pyramidal neurons. *The Journal of neuroscience* 16:5603-5612.
- Brust TB, Cayabyab FS, MacVicar BA (2007) C-Jun N-terminal kinase regulates adenosine A1 receptor-mediated synaptic depression in the rat hippocampus. *Neuropharmacology* 53:906-917.
- Brust TB, Cayabyab FS, Zhou N, MacVicar BA (2006) p38 mitogen-activated protein kinase contributes to adenosine A1 receptor-mediated synaptic depression in area CA1 of the rat hippocampus. *The Journal of neuroscience* 26:12427-12438.
- Burnashev N, Monyer H, Seeburg PH, Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8:189-198.
- Butcher SP, Henshall DC, Teramura Y, Iwasaki K, Sharkey J (1997) Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an antiexcitotoxic mechanism. *The Journal of neuroscience* 17:6939-6946.
- Cayabyab FS, Gowribai K, Walz W (2013) Involvement of matrix metalloproteinases-2 and -9 in the formation of a lacuna-like cerebral cavity. *J Neurosci Res*.
- Chen J-F, Xu K, Petzer JP, Staal R, Xu Y-H, Beilstein M, Sonsalla PK, Castagnoli K, Castagnoli Jr N, Schwarzschild MA (2001) Neuroprotection by caffeine and A (2A) adenosine receptor inactivation in a model of Parkinson's disease. *J Neurosci* 21:143.
- Chen Z, Cherry X, Cassandra P, Jocelyn S, Walz W, Cayabyab FS (2014) Prolonged adenosine A1 receptor activation in hypoxia and pial vessel disruption focal cortical ischemia facilitates clathrin-mediated AMPA receptor endocytosis and long lasting synaptic inhibition in rat hippocampal CA3-CA1 synapses: differential regulation of GluA2 and GluA1 subunits by p38 MAPK and JNK. *Journal of Neuroscience* 34:9621-9643.
- Chu S, Xiong W, Zhang D, Soylu H, Sun C, Albeni BC, Parkinson FE (2013) Regulation of adenosine levels during cerebral ischemia. *Acta Pharmacologica Sinica* 34:60-66.
- Cid C, Garcia-Bonilla L, Camafeita E, Burda J, Salinas M, Alcazar A (2007) Proteomic characterization of protein phosphatase 1 complexes in ischemia-reperfusion and ischemic tolerance. *Proteomics* 7:3207-3218.
- Clem RL, Hagan RL (2010) Calcium-permeable AMPA receptor dynamics mediate fear memory erasure. *science* 330:1108-1112.
- Coelho JE, Rebola N, Fragata I, Ribeiro JA, de Mendonca A, Cunha RA (2006) Hypoxia-induced desensitization and internalization of adenosine A1 receptors in the rat hippocampus. *Neuroscience* 138:1195-1203.
- Collis MG, Hourani SMO (1993) Adenosine receptor subtypes. *Trends in pharmacological sciences* 14:361-366.
- Costenla AR, Diogenes MJ, Canas PM, Rodrigues RJ, Nogueira C, Maroco J, Agostinho PM, Ribeiro JA, Cunha RA, de Mendonca A (2011) Enhanced role of adenosine A(2A) receptors in the modulation of LTP in the rat hippocampus upon ageing. *Eur J Neurosci* 34:12-21.
- Cunha RA (2005) Neuroprotection by adenosine in the brain: from A1 receptor activation to A2A receptor blockade. *Purinergic Signalling* 1:111-134.

- Dale N, Pearson T, Frenguelli BG (2000) Direct measurement of adenosine release during hypoxia in the CA1 region of the rat hippocampal slice. *The Journal of Physiology* 526:143-155.
- De M, Austin KF, Dudley MW (1993) Differential distribution of A3 receptor in rat brain. In: *Soc Neurosci Abstr.*
- de Mendonca A, Ribeiro J (1996) Adenosine and neuronal plasticity. *Life sciences* 60:245-251.
- de Mendonca A, Ribeiro JA (2001) Adenosine and synaptic plasticity. *Drug development research* 52:283-290.
- Delgado JY, Coba M, Anderson CNG, Thompson KR, Gray EE, Heusner CL, Martin KC, Grant SGN, O'Dell TJ (2007) NMDA receptor activation dephosphorylates AMPA receptor glutamate receptor 1 subunits at threonine 840. *The Journal of Neuroscience* 27:13210-13221.
- Dias RB, Ribeiro JA, Sebastiao AM (2012) Enhancement of AMPA currents and GluR1 membrane expression through PKA-coupled adenosine A2A receptors. *Hippocampus* 22:276-291.
- Dias RB, Rombo DM, Ribeiro JA, Sebastião AM (2013) Ischemia-induced synaptic plasticity drives sustained expression of calcium-permeable AMPA receptors in the hippocampus. *Neuropharmacology* 65:114-122.
- Dixon AK, Widdowson L, Richardson PJ (1997) Desensitisation of the adenosine A1 receptor by the A2A receptor in the rat striatum. *Journal of neurochemistry* 69:315-321.
- Don Yi K, Simpkins JW (2008) Protein phosphatase 1, protein phosphatase 2A, and calcineurin play a role in estrogen-mediated neuroprotection. *Endocrinology* 149:5235-5243.
- Dunwiddie TV (1980) Endogenously released adenosine regulates excitability in the in vitro hippocampus. *Epilepsia* 21:541-548.
- Dunwiddie TV, Masino SA (2001) The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci* 24:31-55.
- Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* 28:511-525.
- Espinal J, John Challiss RA, Newsholme EA (1983) Effect of adenosine deaminase and an adenosine analogue on insulin sensitivity in soleus muscle of the rat. *FEBS letters* 158:103-106.
- Esteban JA, Shi S-H, Wilson C, Nuriya M, Huganir RL, Malinow R (2003) PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nature neuroscience* 6:136-143.
- Feoktistov I, Biaggioni I (1997) Adenosine A2B receptors. *Pharmacological Reviews* 49:381-402.
- Feoktistov I, Goldstein AE, Ryzhov S, Zeng D, Belardinelli L, Voyno-Yasenetskaya T, Biaggioni I (2002) Differential expression of adenosine receptors in human endothelial cells role of A2B receptors in angiogenic factor regulation. *Circulation research* 90:531-538.
- Ferré S, Fuxe K, B Fredholm B, Morelli M, Popoli P (1997) Adenosine–dopamine receptor–receptor interactions as an integrative mechanism in the basal ganglia. *Trends in neurosciences* 20:482-487.
- Fredholm BB, Dunwiddie TV (1988) How does adenosine inhibit transmitter release? *Trends in pharmacological sciences* 9:130-134.
- Fredholm BB, Chen J-F, Cunha RA, Svenningsson P, Vaugeois J-M (2005) Adenosine and brain function. *International review of neurobiology* 63:191-270.

- Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W (2000) Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedeberg's archives of pharmacology* 362:364-374.
- Friedman HS, Koroshetz WJ, Qureshi N (1996) Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med* 334:1405.
- Gladstone DJ, Black SE, Hakim AM (2002) Toward wisdom from failure lessons from neuroprotective stroke trials and new therapeutic directions. *Stroke* 33:2123-2136.
- Gorter JA, Petrozzino JJ, Aronica EM, Rosenbaum DM, Opitz T, Bennett MVL, Connor JA, Zukin RS (1997) Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca^{2+} influx in hippocampal CA1 neurons of gerbil. *The Journal of neuroscience* 17:6179-6188.
- Greger IH, Khatri L, Kong X, Ziff EB (2003) AMPA receptor tetramerization is mediated by Q/R editing. *Neuron* 40:763-774.
- Gu JG, Albuquerque C, Lee CJ, MacDermott AB (1996) Synaptic strengthening through activation of Ca^{2+} -permeable AMPA receptors.
- Handa M, Choi DS, Caldeiro RM, Messing RO, Gordon AS, Diamond I (2001) Cloning of a novel isoform of the mouse NBMPR-sensitive equilibrative nucleoside transporter (ENT1) lacking a putative phosphorylation site. *Gene* 262:301-307.
- Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nature neuroscience* 5:405-414.
- He K, Goel A, Ciarkowski CE, Song L, Lee H-K (2011) Brain area specific regulation of synaptic AMPA receptors by phosphorylation. *Commun Integr Biol* 4:569-572.
- Hédou GF, Koshibu K, Farinelli M, Kilic E, Gee CE, Kilic U, Baumgärtel K, Hermann DM, Mansuy IM (2008) Protein phosphatase 1-dependent bidirectional synaptic plasticity controls ischemic recovery in the adult brain. *The Journal of Neuroscience* 28:154-162.
- Hershfield MS (2005) New insights into adenosine-receptor-mediated immunosuppression and the role of adenosine in causing the immunodeficiency associated with adenosine deaminase deficiency. *European journal of immunology* 35:25-30.
- Hogan YH, Hawkins R, Alkadhi KA (1998) Adenosine A1 receptor activation inhibits LTP in sympathetic ganglia. *Brain research* 807:19-28.
- Hua R, Walz W (2006) Minocycline treatment prevents cavitation in rats after a cortical devascularizing lesion. *Brain research* 1090:172-181.
- Huang CC, Liang YC, Hsu KS (1999) A role for extracellular adenosine in time-dependent reversal of long-term potentiation by low-frequency stimulation at hippocampal CA1 synapses. *The Journal of neuroscience* 19:9728-9738.
- Ikonomidou C, Turski L (2002) Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *The Lancet Neurology* 1:383-386.
- Ito M, Karachot L (1992) Protein kinases and phosphatase inhibitors mediating long-term desensitization of glutamate receptors in cerebellar Purkinje cells. *Neuroscience research* 14:27-38.
- Jacobson KA, Nikodijević O, Shi D, Gallo-Rodriguez C, Olah ME, Stiles GL, Daly JW (1993) A role for central A3 adenosine receptors: Mediation of behavioral depressant effects. *FEBS letters* 336:57-60.
- Jennings K (2001) Am I doing a higher grade's work? *Nurs Times* 97:23.
- Kim GS, Jung JE, Niizuma K, Chan PH (2009) CK2 is a novel negative regulator of NADPH oxidase and a neuroprotectant in mice after cerebral ischemia. *J Neurosci* 29:14779-14789.

- Kiss A, Farah K, Kim J, Garriock RJ, Drysdale TA, Hammond JR (2000) Molecular cloning and functional characterization of inhibitor-sensitive (mENT1) and inhibitor-resistant (mENT2) equilibrative nucleoside transporters from mouse brain. *Biochem J* 352 Pt 2:363-372.
- Kocsis JD, Eng DL, Bhisitkul RB (1984) Adenosine selectively blocks parallel-fiber-mediated synaptic potentials in rat cerebellar cortex. *Proceedings of the National Academy of Sciences* 81:6531-6534.
- Koh P-O (2011) Focal Cerebral Ischemia Reduces Protein Phosphatase 2A Subunit B Expression in Brain Tissue and HT22 Cells. *Laboratory animal research* 27:73-76.
- Kong W, Engel K, Wang J (2004) Mammalian nucleoside transporters. *Curr Drug Metab* 5:63-84.
- Kristensen BW, Noraberg J, Zimmer J (2001) Comparison of excitotoxic profiles of ATPA, AMPA, KA and NMDA in organotypic hippocampal slice cultures. *Brain research* 917:21-44.
- Kwak S, Weiss JH (2006) Calcium-permeable AMPA channels in neurodegenerative disease and ischemia. *Current opinion in neurobiology* 16:281-287.
- Laghi Pasini F, Guideri F, Picano E, Parenti G, Petersen C, Varga A, Di Perri T (2000) Increase in plasma adenosine during brain ischemia in man: a study during transient ischemic attacks, and stroke. *Brain research bulletin* 51:327-330.
- Lammie GA (2000) Pathology of small vessel stroke. *British medical bulletin* 56:296-306.
- Largo C, Cuevas P, Herreras O (1996) Is glia disfunction the initial cause of neuronal death in ischemic penumbra? *Neurological research* 18:445-448.
- Latini S, Pazzagli M, Pepeu G, Pedata F (1996a) A2 adenosine receptors: their presence and neuromodulatory role in the central nervous system. *General Pharmacology: The Vascular System* 27:925-933.
- Latini S, Corsi C, Pedata F, Pepeu G (1996b) The source of brain adenosine outflow during ischemia and electrical stimulation. *Neurochemistry international* 28:113-118.
- Launey T, Endo S, Sakai R, Harano J, Ito M (2004) Protein phosphatase 2A inhibition induces cerebellar long-term depression and declustering of synaptic AMPA receptor. *Proceedings of the National Academy of Sciences of the United States of America* 101:676-681.
- Lee H-K, Takamiya K, He K, Song L, Huganir RL (2010) Specific roles of AMPA receptor subunit GluR1 (GluA1) phosphorylation sites in regulating synaptic plasticity in the CA1 region of hippocampus. *Journal of Neurophysiology* 103:479-489.
- Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, Huganir RL (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* 112:631-643.
- Lee JH, Kim KY, Lee Y-K, Park SY, Kim CD, Lee WS, Rhim BY, Hong KW (2004) Cilostazol prevents focal cerebral ischemic injury by enhancing casein kinase 2 phosphorylation and suppression of phosphatase and tensin homolog deleted from chromosome 10 phosphorylation in rats. *Journal of Pharmacology and Experimental Therapeutics* 308:896-903.
- Li D-P, Pan H-L (2013) CK1 regulates NMDA receptor activity through protein phosphatase-1 in hypothalamic presympathetic neurons in hypertension. *The FASEB Journal* 27:697-618.
- Lin JW, Ju W, Foster K, Lee SH, Ahmadian G, Wyszynski M, Wang YT, Sheng M (2000) Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization. *Nature neuroscience* 3:1282-1290.

- Linden J (2001) Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annual review of pharmacology and toxicology* 41:775-787.
- Litchfield D (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J* 369:1-15.
- Liu B, Liao M, Mielke JG, Ning K, Chen Y, Li L, El-Hayek YH, Gomez E, Zukin RS, Fehlings MG (2006) Ischemic insults direct glutamate receptor subunit 2-lacking AMPA receptors to synaptic sites. *The Journal of neuroscience* 26:5309-5319.
- Liu SJ, Zukin RS (2007) Ca²⁺-permeable AMPA receptors in synaptic plasticity and neuronal death. *Trends in neurosciences* 30:126-134.
- Lu W, Roche KW (2012) Posttranslational regulation of AMPA receptor trafficking and function. *Current opinion in neurobiology* 22:470-479.
- Lu YM, Yin HZ, Chiang J, Weiss JH (1996) Ca²⁺-permeable AMPA/kainate and NMDA channels: high rate of Ca²⁺ influx underlies potent induction of injury. *The Journal of neuroscience* 16:5457-5465.
- MacDonald RL, Skerritt JH, Werz MA (1986) Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurones in cell culture. *The Journal of physiology* 370:75-90.
- Mann J (2002) Efficacy of Tissue Plasminogen Activator (Tpa) for Stroke: Truths about the NINDS study: setting the record straight. *Western journal of medicine* 176:192.
- Marler JR, Tilley BC, Lu M, Brott TG, Lyden PC, Grotta JC, Broderick JP, Levine SR, Frankel MP, Horowitz SH (2000) Early stroke treatment associated with better outcome The NINDS rt-PA Stroke Study. *Neurology* 55:1649-1655.
- Martin L, Blackstone C, Levey A, Huganir R, Price D (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. *Neuroscience* 53:327.
- Mulkey RM, Herron CE, Malenka RC (1993) An essential role for protein phosphatases in hippocampal long-term depression. *Science* 261:1051-1055.
- Murakami K, Kondo T, Kawase M, Li Y, Sato S, Chen SF, Chan PH (1998) Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *The journal of neuroscience* 18:205-213.
- Nagy LE, Diamond I, Casso DJ, Franklin C, Gordon AS (1990) Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J Biol Chem* 265:1946-1951.
- Nakagawa T, Cheng Y, Ramm E, Sheng M, Walz T (2005) Structure and different conformational states of native AMPA receptor complexes. *Nature* 433:545-549.
- Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* 258:597-603.
- Noh K-M, Yokota H, Mashiko T, Castillo PE, Zukin RS, Bennett MVL (2005) Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. *Proceedings of the National Academy of Sciences of the United States of America* 102:12230-12235.
- Noji T, Karasawa A, Kusaka H (2004) Adenosine uptake inhibitors. *Eur J Pharmacol* 495:1-16.
- Nuydens R, De Jong M, Van Den Kieboom G, Heers C, Dispersyn G, Cornelissen F, Nuyens R, Borgers M, Geerts H (1998) Okadaic Acid-Induced Apoptosis in Neuronal Cells: Evidence for an Abortive Mitotic Attempt. *Journal of neurochemistry* 70:1124-1133.
- Obrietan K, Belousov AB, Heller HC, van den Pol AN (1995) Adenosine pre-and postsynaptic modulation of glutamate-dependent calcium activity in hypothalamic neurons. *Journal of neurophysiology* 74:2150-2162.

- Ochiishi T, Chen L, Yukawa A, Saitoh Y, Sekino Y, Arai T, Nakata H, Miyamoto H (1999) Cellular localization of adenosine A1 receptors in rat forebrain: immunohistochemical analysis using adenosine A1 receptor-specific monoclonal antibody. *J Comp Neurol* 411:301-316.
- Oh MC, Derkach VA (2005) Dominant role of the GluR2 subunit in regulation of AMPA receptors by CaMKII. *Nat Neurosci* 8:853-854.
- Oh MC, Derkach VA, Guire ES, Soderling TR (2006) Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation. *Journal of Biological Chemistry* 281:752-758.
- Pantoni L (2010) Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. *The Lancet Neurology* 9:689-701.
- Parkinson FE, Damaraju VL, Graham K, Yao SYM, Baldwin SA, Cass CE, Young JD (2011) Molecular biology of Nucleoside transporters and their distributions and functions in the brain. *Current Topics in Medicinal Chemistry* 11:948-972.
- Pearson T, Nuritova F, Caldwell D, Dale N, Frenguelli BG (2001) A depletable pool of adenosine in area CA1 of the rat hippocampus. *The Journal of neuroscience* 21:2298-2307.
- Pedata F, Pugliese AM, Corti F, Melani A (2013) Adenosine and Stroke. In: *Adenosine*, pp 273-306: Springer.
- Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS (1997) The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. *Trends Neurosci* 20:464-470.
- Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Pulsinelli WA (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proceedings of the National Academy of Sciences* 89:10499-10503.
- Plant K, Pelkey KA, Bortolotto ZA, Morita D, Terashima A, McBain CJ, Collingridge GL, Isaac JTR (2006) Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. *Nature neuroscience* 9:602-604.
- Pugliese AM, Traini C, Cipriani S, Gianfriddo M, Mello T, Giovannini MG, Galli A, Pedata F (2009) The adenosine A2A receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices. *British journal of pharmacology* 157:818-830.
- Rebholz H, Nishi A, Liebscher S, Nairn AC, Flajolet M, Greengard P (2009) CK2 negatively regulates Gas signaling. *Proceedings of the National Academy of Sciences* 106:14096-14101.
- Rebola N, Lujan R, Cunha RA, Mulle C (2008) Adenosine A2A receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. *Neuron* 57:121-134.
- Rebola N, Rodrigues R, Lopes L, Richardson P, Oliveira C, Cunha R (2005) Adenosine A1 and A2A receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. *Neuroscience* 133:79-83.
- Ribeiro JA (2005) What can adenosine neuromodulation do for neuroprotection? *Current Drug Targets-CNS & Neurological Disorders* 4:325-329.
- Roche KW, O'Brien RJ, Mammen AL, Bernhardt J, Huganir RL (1996) Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* 16:1179-1188.
- Rogers S, Hughes T, Hollmann M, Gasic G, Deneris E, Heinemann S (1991) The characterization and localization of the glutamate receptor subunit GluR1 in the rat brain. *The Journal of neuroscience* 11:2713-2724.

- Rudolph KA, Schubert P (1995) Adenosine and brain ischemia. In: Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology, pp 391-397: Springer.
- Rudolph KA, Schubert P, Parkinson FE, Fredholm BB (1992) Neuroprotective role of adenosine in cerebral ischaemia. *Trends in pharmacological sciences* 13:439-445.
- Rundén E, Seglen PO, Haug F-M, Ottersen OP, Wieloch T, Shamloo M, Laake JH (1998) Regional selective neuronal degeneration after protein phosphatase inhibition in hippocampal slice cultures: evidence for a MAP kinase-dependent mechanism. *The Journal of neuroscience* 18:7296-7305.
- Sanderson G, Scholfield CN (1986) Effects of adenosine uptake blockers and adenosine on evoked potentials of guinea-pig olfactory cortex. *Pflügers Arch* 406:25-30.
- Schiff SJ, Somjen GG (1987) The effect of graded hypoxia on the hippocampal slice: an in vitro model of the ischemic penumbra. *Stroke* 18:30-37.
- Sebastião AM, de Mendonça A, Ribeiro JA (2001) Neuroprotection during hypoxic insults: role of adenosine. *Drug development research* 52:291-295.
- Sharkey J, Butcher SP (1994) Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia.
- Shen H-Y, Chen J-F (2009) Adenosine A2A receptors in psychopharmacology: modulators of behavior, mood and cognition. *Current neuropharmacology* 7:195.
- Shen H-Y, Lusardi TA, Williams-Karnesky RL, Lan J-Q, Poulsen DJ, Boison D (2011) Adenosine kinase determines the degree of brain injury after ischemic stroke in mice. *Journal of Cerebral Blood Flow & Metabolism* 31:1648-1659.
- Shintani-Ishida K, Yoshida K-i (2011) Ischemia induces phospholamban dephosphorylation via activation of calcineurin, PKC- α , and protein phosphatase 1, thereby inducing calcium overload in reperfusion. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1812:743-751.
- Shryock JC, Snowdy S, Baraldi PG, Cacciari B, Spalluto G, Monopoli A, Ongini E, Baker SP, Belardinelli L (1998) A2A-adenosine receptor reserve for coronary vasodilation. *Circulation* 98:711-718.
- Sichardt K, Nieber K (2007) Adenosine A₁ receptor: Functional receptor-receptor interactions in the brain. *Purinergic signalling* 3:285-298.
- Sirén A-L, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, Keenan S, Gleiter C, Pasquali C, Capobianco A (2001) Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proceedings of the National Academy of Sciences* 98:4044-4049.
- Sommer C, Kiessling M (2002) Ischemia and Ischemic Tolerance Induction Differentially Regulate Protein Expression of GluR1, GluR2, and AMPA Receptor Binding Protein in the Gerbil Hippocampus GluR2 (GluR-B) Reduction Does Not Predict Neuronal Death. *Stroke* 33:1093-1100.
- Sommer C, Schomacher M, Berger C, Kuhnert K, Müller HD, Schwab S, Schäbitz W-R (2006) Neuroprotective cannabinoid receptor antagonist SR141716A prevents downregulation of excitotoxic NMDA receptors in the ischemic penumbra. *Acta neuropathologica* 112:277-286.
- Stolk M, Cooper E, Vilk G, Litchfield DW, Hammond JR (2005) Subtype-specific regulation of equilibrative nucleoside transporters by protein kinase CK2. *Biochem J* 386:281-289.
- Szabo A, Somogyi J, Cauli B, Lambolez B, Somogyi P, Lamsa KP (2012) Calcium-permeable AMPA receptors provide a common mechanism for LTP in glutamatergic synapses of distinct hippocampal interneuron types. *The Journal of neuroscience* 32:6511-6516.

- Traystman RJ (2003) Animal models of focal and global cerebral ischemia. *ILAR J* 44:85-95.
- Trussell LO, Jackson MB (1985) Adenosine-activated potassium conductance in cultured striatal neurons. *Proceedings of the National Academy of Sciences* 82:4857-4861.
- Uchino H, Minamikawa-Tachino R, Kristián T, Perkins G, Narazaki M, Siesjö BK, Shibasaki F (2002) Differential neuroprotection by cyclosporin A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. *Neurobiology of disease* 10:219-233.
- Van Wylen DG, Park TS, Rubio R, Berne RM (1986) Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia. *J Cereb Blood Flow Metab* 6:522-528.
- Vandenberghe W, Robberecht W, Brorson JR (2000) AMPA receptor calcium permeability, GluR2 expression, and selective motoneuron vulnerability. *The Journal of neuroscience* 20:123-132.
- Walsh AH, Cheng A, Honkanen RE (1997) Fostriecin, an antitumor antibiotic with inhibitory activity against serine/threonine protein phosphatases types 1 (PP1) and 2A (PP2A), is highly selective for PP2A. *FEBS letters* 416:230-234.
- Wang K, Walz W (2003) Unusual topographical pattern of proximal astrogliosis around a cortical devascularizing lesion. *Journal of neuroscience research* 73:497-506.
- Wang K, Bekar LK, Furber K, Walz W (2004) Vimentin-expressing proximal reactive astrocytes correlate with migration rather than proliferation following focal brain injury. *Brain research* 1024:193-202.
- Wardas J (2002) Neuroprotective role of adenosine in the CNS. *Polish journal of pharmacology* 54:313-326.
- Yan Z, Hsieh-Wilson L, Feng J, Tomizawa K, Allen PB, Fienberg AA, Nairn AC, Greengard P (1999) Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. *Nature neuroscience* 2:13-17.
- Ye Z-Y, Li D-P, Li L, Pan H-L (2011) Protein kinase CK2 increases glutamatergic input in the hypothalamus and sympathetic vasomotor tone in hypertension. *The Journal of Neuroscience* 31:8271-8279.
- Yoon KW, Rothman SM (1991) Adenosine inhibits excitatory but not inhibitory synaptic transmission in the hippocampus. *The Journal of neuroscience* 11:1375-1380.
- Zhang C, Schmidt JT (1998) Adenosine A1 receptors mediate retinotectal presynaptic inhibition: uncoupling by C-kinase and role in LTP during regeneration. *Journal of neurophysiology* 79:501-510.
- Zhang D, Xiong W, Albeni BC, Parkinson FE (2011) Expression of human equilibrative nucleoside transporter 1 in mouse neurons regulates adenosine levels in physiological and hypoxic-ischemic conditions. *Journal of neurochemistry* 118:4-11.
- Zhao X, Yeh JZ, Narahashi T (2001) Post-Stroke Dementia. *Annals of the New York Academy of Sciences* 939:179-186.
- Zhou Q-Y, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O (1992) Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proceedings of the National Academy of Sciences* 89:7432-7436.
- Zur Nedden S, Hawley S, Pentland N, Hardie DG, Doney AS, Frenguelli BG (2011) Intracellular ATP influences synaptic plasticity in area CA1 of rat hippocampus via metabolism to adenosine and activity-dependent activation of adenosine A1 receptors. *The Journal of neuroscience* 31:6221-6234.